

AN EMBRYOLOGICAL STUDY OF HARELIP IN MICE

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SEVEN FIGURES

In a previous publication by the writer and G. D. Snell, it has been shown that harelip in mice is inherited as a recessive mendelian character subject to some variation in expression. The purpose of the present paper is to describe the steps by which this anatomical anomaly becomes established in the development of the mouse.

Harelip, similar to that of the mouse, occurs in a small percentage of human beings, and in swine, dogs, and some other animals. There can be little doubt that, in many of these cases, as in that of the mouse, heredity is involved in the genesis of the defect. Hence the interest which attaches to its further investigation.

In this study timed embryos were obtained by the following procedure. Mice of opposite sex, which had been previously tested and found to produce a high percentage of harelip and cleft palate young, were allowed to remain together for 1 hour each day. The female was then inspected for the presence of a vaginal plug. If a plug was present, the female was considered to have been impregnated and was later killed when the embryos had attained the age desired.

The embryos were fixed in either Bouin's picro-formol, or Zenker's fluid, sectioned, and stained in 1 of 3 different stain combinations, viz., a) Mallory's triple stain, b) haemalum and light green, or, c) Delafield's haematoxylin and eosin. The most satisfactory treatment was as follows; 2 minutes

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in Delafield's, prolonged washing in tap water, 35 per cent alcohol for 2 minutes and then 1 minute in eosin Y. All sections were serial, cut in the transverse plane, and were $10\ \mu$ thick.

The drawings included in this paper, with the exception of figure 1, were made with the aid of the camera lucida and are all of the same magnification and reduction. The animals studied were either embryos or newly born. The age of embryos is reckoned from the hour of copulation of the parents. At birth the lip has not differentiated from the jaw tissue; thus the term 'harelip' as used here means a cleft in the upper jaw.

In orientation the words 'dorsal' and 'ventral' are used in this sense; dorsal is the back or pharynx end of the mouth and ventral the front or receptive portion.

The youngest embryos studied were exactly 10 days old, reckoning from the time of vaginal plug formation. At this stage little differentiation of nasal structures had occurred.

The course of development during the latter part of the tenth day and the beginning of the eleventh day is indicated by a litter (series Q) in which the five embryos form a graded series of developmental stages, referable perhaps to slightly different time intervals between the beginning of development in each embryo. For in the mouse, rupture of the Graafian follicles may not be in all cases simultaneous in the same oestrous cycle. Thus the embryos of a single litter may differ by as much as several hours in developmental age.

The first indication of abnormality is in connection with the formation of the nasal fossae. Normally, this is accomplished by the growth and fusion of the lateral nasal processes with the mesial nasal processes. Various stages in this process are shown in figures 2 to 4.

Figure 1 shows the head of an embryo of 10 days and 18 hours. This figure is for general orientation purposes and shows the approximate stage of development of the embryos from which the sections shown in figures 2 to 4 were taken.

Figure 2 shows a transverse section through the head in the region of the nasal placode and in the plane x-x of figure 1.

Figure 3 shows a similar section of an embryo of the same litter but slightly more advanced in development. The nasal placode has become an open groove by the development of the lateral and mesial nasal processes.

Figure 4 is from the developmentally oldest member of the litter. At the point where this section was taken fusion of

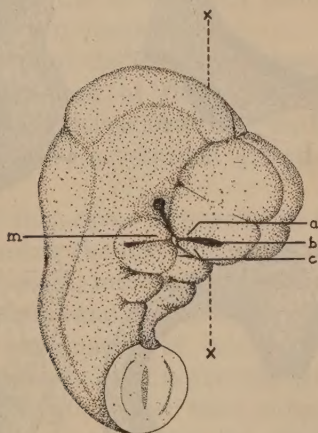


Fig. 1 Head of a normal embryo of 10 days 18 hours. Note the olfactory pit and the nasal processes on each side. The line x-x shows the plane through the head at which all the sections were taken. *a*, lateral nasal process; *b*, olfactory pit; *c*, mesial nasal process; *m*, maxillary process.

the approximated processes has occurred. The fusion of these processes commences dorsally at the back of the mouth and proceeds ventrad to the front.

At the same time that the mesial nasal process is fusing with the internal surface of the lateral nasal process, the maxillary process is fusing with the external surface of the lateral nasal process. The point of fusion between the two nasal processes is always about 100μ in front of (ventrad to) the point of fusion between the maxillary and lateral nasal processes.

The work of Glas ('04, fig. 13) shows the fusion of the lateral and mesial nasal processes in the normal rat.

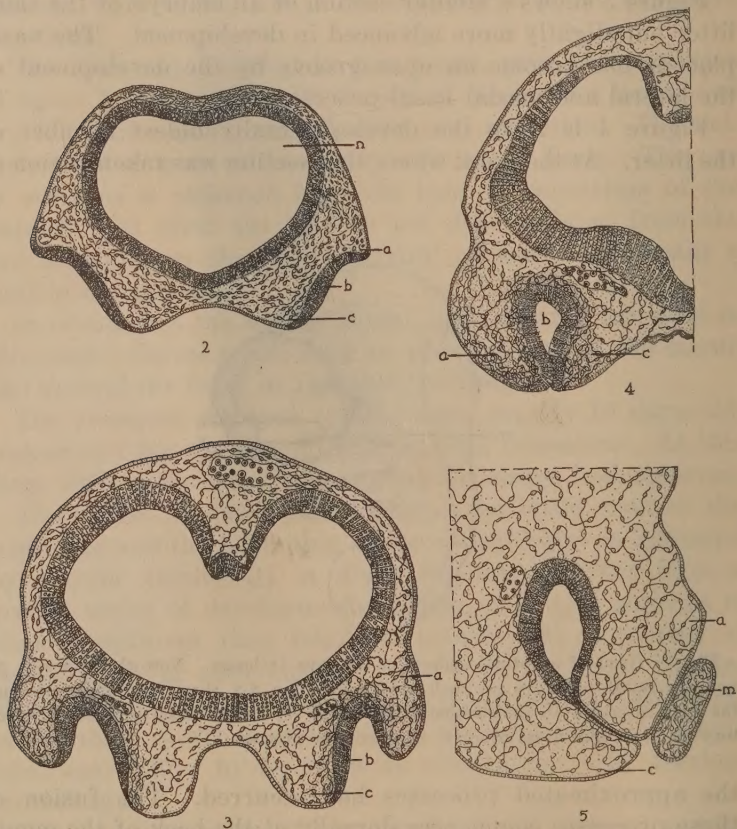


Fig. 2 Transverse section through an embryo head at about the same developmental stage as the embryo in figure 1. *a*, lateral nasal process; *b*, nasal placode and pit; *c*, mesial nasal process; *n*, neural tube.

Fig. 3 Transverse section at the same region as in figure 2. *a*, lateral nasal process; *b*, nasal placode; *c*, mesial nasal process.

Fig. 4 Section from a normal embryo of the same litter as the previous figures. One side of the section omitted from the diagram for convenience. *a*, lateral nasal process; *b*, primitive nasal fossa; *c*, mesial nasal process.

Fig. 5 Portion of a transverse section of the left nasal region of a normal embryo N7 (11 days 14½ hours), showing fusion of the lateral nasal process (*a*); with the mesial nasal process (*c*); and its relation to the tip of the maxillary process (*m*).

The situation, as concerns the nasal fossae, when the lateral and mesial nasal processes fail to fuse is presented in figure 6. From the time of vaginal plug formation this embryo is $8\frac{1}{2}$ hours older than the normal embryo shown in figure 4. Developmentally, this embryo is correspondingly older, yet fusion of the nasal processes has not occurred; in five sibs of the same size and development fusion has been completed. A complete intergradation between the situation shown in figure 6 and that in the newly born mouse with harelip is found in the writer's preparations.

Along with failure of the nasal processes to fuse with each other, there is also lack of fusion between the maxillary process and the mesial nasal process. Both of these deficiencies are attributable to a retarded growth of the maxillary process.

We have found that cases of harelip in the mouse may be first discovered during the last few hours of the tenth day of embryonic development, indicated by failure of fusion to occur between the lateral and mesial nasal processes. We must now look for an explanation of this failure. The first explanation which presents itself is that the lateral nasal process may have a growth rate slower than normal, thus failing to make the necessary contact with the mesial nasal process. No evidence of this was found, the lateral nasal processes apparently having sufficient bulk in all cases; all that would be necessary to bring about contact would be pressure on their external surfaces.

This observation led the writer to the idea that in normal embryos the necessary pressure is supplied by the growth of the maxillary processes external to the lateral nasal processes. The maxillary processes are growing toward the median plane and find the lateral nasal processes in their way; naturally they must force these lateral processes toward the median plane and out of their way. This pressure would account for the contact normally made between the lateral and mesial nasal processes. If the growth of the maxillary processes is for any reason retarded, the delicate pressure relationships

will be upset; the maxillary process upon finally acquiring normal size at a particular point will find the normally developed lateral nasal process able to resist its pressure at that point.

Such pressure is produced by growth of the maxillary processes in the normal pig and is described by Patten ('31,

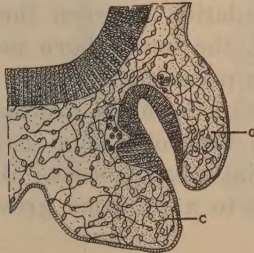


Fig. 6 Section of a harelip embryo (V6, 11 days 2½ hours) in which the lateral nasal and mesial nasal processes have failed to fuse. *a*, lateral nasal process; *c*, mesial nasal process.

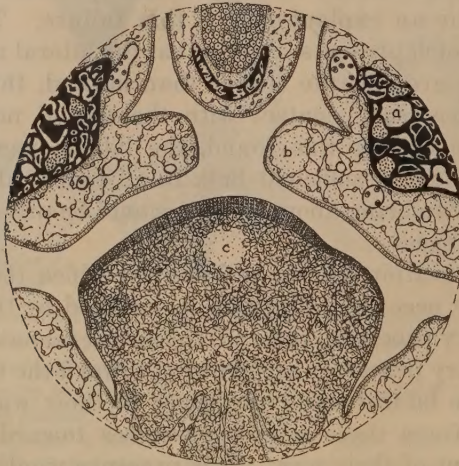


Fig. 7 Part of a transverse section in the palatal region of embryo AC2 (19 days old). *a*, calcified portion of alveolar process of the maxilla; *b*, horizontal palatine process; *c*, tongue. At the top, center, may be seen the cartilaginous nasal septum and the calcified vomer bone.

p. 281) thus, "The maxillary processes are much more prominent and have grown toward the midline, crowding the nasal processes closer to each other."

Figure 5 shows the relationship of the lateral nasal process to the mesial nasal and the maxillary processes when fusion occurs. It may be seen that at the point where fusion occurs the maxillary process is very slender, even in normal embryos.

In harelip mice some fusion occurs between the lateral and mesial nasal processes in all cases, though it seldom proceeds ventrad of the usual site of the posterior nares.

Measurements of the floors (fused portions) of the nasal fossae in harelip and normal embryos were taken. These show a floor length of 90μ in harelip mice and of 320μ in normal mice of 11 days $2\frac{1}{2}$ hours. At 13 days the floors of the nasal fossae in normal embryos have increased their length to over 1000μ . This length includes about 400μ of opening due to the posterior nares. The posterior nares have broken through the floors of the nasal fossae in the normal embryos, but in the harelip embryos the floors at this stage have not increased in length since the 11-day stage, and have not grown forward (ventrad) into the region at which the posterior nares normally break through.

If we now examine the length of the floor of the nasal fossa in newly born mice, we find that, due to the great increase in size of all parts of the head, it has attained an absolute length of approximately 800μ , though its relative length is unchanged. The floor of the nasal fossa of the normal newly born mouse has meanwhile attained a length of about 4300μ .

In normal embryos the upper jaw arises from the fused lateral nasal, mesial nasal, and maxillary processes. When fusion of these processes fails, differentiation on each side of the cleft or clefts proceeds as best it may, the result, in regard to bone structure, being shown in figure 1 of Reed and Snell ('31).

The second critical stage in the development of harelip and cleft palate in mice occurs during formation of the definitive palate. The palatine processes arise from the mesial sides

of the maxillary processes and at first hang almost vertically in the buccal cavity on each side of the tongue. They are then rotated into the horizontal plane either by unequal growth or by muscular contraction. After rotation has occurred, growth in the horizontal plane continues until the palatal plates come in contact with each other and fuse. The position of the palatine processes immediately after rotation in normal human embryos is illustrated in figure 7 of Sicher ('15). In harelip mice rotation occurs, but is retarded to such an extent that in normal litter mates rotation, horizontal growth, and fusion have all taken place before rotation occurs in the abnormal embryos.

After rotation in harelip mice growth in the horizontal plane may take place to a limited extent, but is seldom sufficient to cause contact in the vicinity of the median plane. The consequent cleft is designated as cleft palate. Figure 7 represents this situation in which rotation of the palatine processes has occurred, but horizontal growth is incomplete.

It appears, then, that cleft palate is due to a retarded growth of the palatine portions of the maxillary processes. We have seen that harelip is also due to a defect in the proper growth of the maxillary process. Both conditions may be considered the result of a retarded growth rate of the maxillary processes. Harelip and cleft palate in mice are both due to the same genetic basis, though possibly with different genetic modifiers; thus the agreement between genetic and embryological observations is excellent.

The data presented in this paper will perhaps be of some interest to the oral surgeon. Dr. V. P. Blair is the author of an excellent book, "Surgery and diseases of the mouth and jaw." He lists the principal theories for failure of fusion of the various processes. He recognizes that heredity is a method of transmission of the character. In mice it would seem that heredity is the only method of transmission of the cause of the abnormality.

Some of the theories which attempt to explain the presence of harelip and cleft palate are:

1. The theory of Warnekros which claims that clefts may be due to supernumerary teeth. The work on mice gives no support to this theory, and the writer would suggest that it further discredits the theory. At the time when evidences of harelip first appear the structures concerned with it could be little affected by the number of teeth which will later arise. In sections and Spalteholtz preparations of newly born mice the normal number of teeth has been observed in each case without regard to the type of cleft.

2. Failure of the tongue to recede from between the palate folds has been considered a cause of the abnormality. But harelip is detectable before the tongue becomes a factor in the case, and the tongue withdraws from the palate by the time the abnormal palatine processes are ready to grow in the horizontal plane.

3. Malnutrition as an explanation of harelip and cleft palate has had few supporters. Several mice were killed before parturition and the positions of harelip and normal embryos in the horns of the uteri were recorded. No correlation between condition of the mouth and position of the embryo could be established.

4. Admittedly under the influence of his own work, which is the first attempt made to investigate the embryology of harelip and cleft palate with satisfactory material, the writer presents the thesis that this interesting abnormality is due to a retarded growth rate of portions of the maxillary processes.

SUMMARY

An hereditary abnormality in mice, harelip and cleft palate, has been investigated embryologically. Clefts of the jaw (harelip) are due primarily to the failure of the lateral nasal processes and the mesial nasal processes to fuse. This failure is probably in turn due to a retarded growth rate of the maxillary processes which fail to exert needed lateral pressure at the critical time. Failure of the palatine processes to unite in the midsagittal plane or with the nasal septum is

due to the retarded growth of the region of the maxillary processes from which they originate.

An explanation for this retarded growth presents an interesting problem in the localized effect of a gene.

It is hoped that the results presented may be of interest to the specialist in oral surgery and to the general practitioner as well as to the anatomist and embryologist.

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THE INNERVATION OF THE DIGESTIVE TRACT IN THE 6-DAY CHICK EMBRYO

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FOUR FIGURES

The organogenesis of the abdominal autonomic nervous system in the chick embryo is nearly completed around the sixth day of development. At that age the various sets of nervous elements have reached their terminal organs and the pattern of distribution of the autonomic innervation of the digestive tract has been established. As a basis for further studies on the histogenetic development of the intravisceral nervous plexuses and ganglia, accurate reconstructions of the extrinsic innervation of the digestive tract are needed.¹

A preliminary attempt at such a reconstruction, completed while the writer was still a member of the department of anatomy at Yale University, is herewith presented. It is based upon a 5-day embryo stained by the Bielschowsky-Paton silver impregnation method. Consecutive serial sections were drawn with the Edinger projecting apparatus and then the central nervous system, the digestive tract, and its extrinsic nerves were modeled in wax. Considerable difficulty was encountered in the modeling of tiny nerve branches; their plexus-like arrangement with anastomotic bundles of very few nerve fibers, the relative thickness of the sections (10μ), and the inevitable loss of a certain amount of tissue between two consecutive sections made it very difficult to give an ac-

¹ For literature dealing with morphological and experimental observations on the enteric nervous system, see previous papers describing the early development of the autonomic nervous system in chick embryos (1931-1932).

curate representation of the distribution of the nervous branches to the various levels of the digestive tract.

A drawing of the first model is shown in figure 1. The pneumogastric nerves extend as far as the upper part of the stomach and they form a very rich plexus surrounding the esophagus and the bronchi; there is not yet any branch coming from the sympathetic chains to these organs. The caudal part of the stomach, the anterior intestine, and the pancreas receive numerous nervous branches deriving from the sympathetic chains; the posterior intestine shows the intestinal nerve of Remak, which extends anteriorly and mingles its

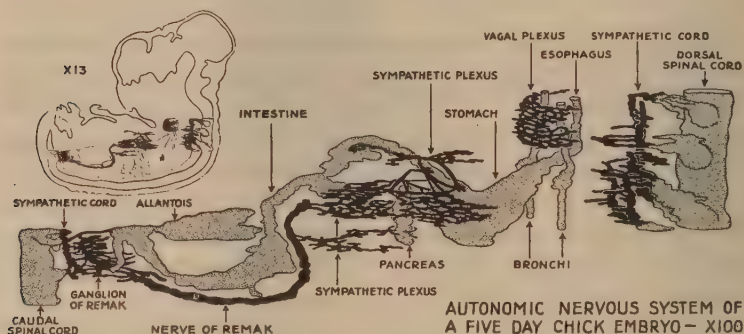


Fig. 1 Wax reconstruction of the extrinsic nerves of the gastro-intestinal tract of a 5-day chick embryo.

terminal fibers with the previously mentioned anterior intestine branches.

In the 6-day embryo, the extremely rich distribution of nervous bundles, too small to be modeled with wax, made it necessary to devise a more accurate way of reconstruction—a combined wax reconstruction and graphic representation. This embryo had also been impregnated following the Bielschowsky-Paton method.

Outlines of the spinal cord, the sympathetic chains, and the digestive tract in every section were obtained by throwing the image of the section on the focusing screen of a photomicrographic apparatus and drawing the image from the

latter. Then, wax preparations of each individual drawing were made, arranged in a serial order; by piling together of these wax preparations, the entire model of the above mentioned organs was reconstructed; this model is measuring about 1 foot. The assembled model has been photographed from the right side, i.e., the right side of the embryo. A lantern slide was made from this photograph and it was projected on a paper 6 feet wide; the outlines of the spinal cord, the sympathetic chains, and the gastro-intestinal tract were drawn. The drawing was then divided transversally in such a way that each divided segment corresponded to a microscopical section in a serial order. The visceral nerve fibers of each individual section were drawn in the corresponding segment. This reconstruction drawing gave us an accurate and complete representation of the extrinsic nerve supply of the digestive tract.

A photograph of this plate is shown in figure 2; the spinal cord (Sp.C.), and the various levels of the digestive tract (E., S., D., S., I., L., I.) are shown in outline; the pancreas (P.) and the umbilical pedicle (O.P.) are shown by initials.

A detailed topographic description of all the nervous branches would be of doubtful interest, and we will only emphasize the main facts about the innervation of the various levels of the digestive tract.

THE ESOPHAGUS

The vagus nerves (L.V., R.V.) run all along the esophagus, on either side of the organ and near its ventral aspect. From the main trunks, numerous smaller branches are given off and form a rich plexus surrounding the esophagus; this plexus is mostly myenteric, although delicate fibers run in the sub-mucous layer.

From this periesophageal plexus arise branches which surround the bronchi, penetrate into the hilus of the lungs, and form intrapulmonary nervous ramifications. Ventrally, both vagi and the branches which connect them give numerous fiber bundles which reach the heart on the mesial aspect of

the sinus venosus and they can be followed for a certain distance in the walls of the atrium.

In this esophageal region, the sympathetic chains (Sy.C.) give rise to numerous nervous branches; many fibers reach the esophageal wall, mix with the vagal plexus, and from

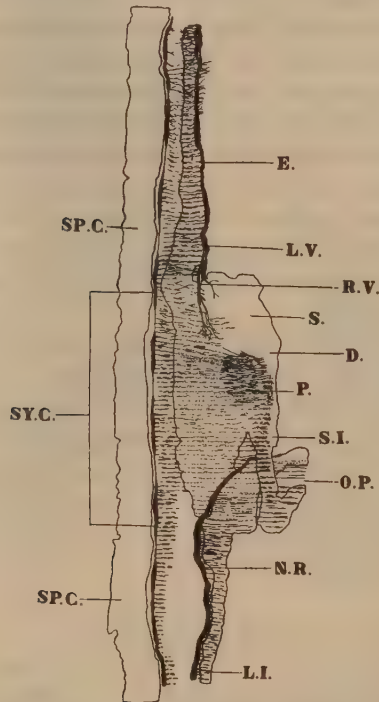


Fig.2 Graphic representation of the extrinsic nerves of the gastro-intestinal tract of a 6-day embryo.

this point on, they cannot be distinguished from the vagal fibers.

The esophagus is, then, innervated by the vagi and by branches from the sympathetic chains; the latter branches are secondary, since in younger stages only vagal fibers are found.

THE STOMACH

The main trunks of both vagus nerves, after having exchanged numerous anastomotic branches, pass along the right side of the esophageal opening of the stomach. The left aspect of the stomach receives only smaller branches deriving from both vagi, but mainly from the left one. Owing to the postero-anterior orientation of the upper part of the organ, both nerves are found in the obtuse angle between the gizzard and the proventriculus. The nerves divide into smaller and smaller branches which are located between the serosa and the muscular layer. From these subserous branches, arise numerous bundles penetrating into the muscular layers and forming a richly developed myenteric plexus.

To this myenteric plexus, fibers from the sympathetic chains contribute to a marked extent; most of them join the vagal plexus near the opening of the esophagus in the stomach.

In the plate, the vagal and the sympathetic innervation have been drawn in the upper part of the stomach only, cephalic to the pancreas, in order to show the innervation of the other organs without the inevitable overlapping of nerve trunks when seen from the right side.

THE ANTERIOR INTESTINE AND THE PANCREAS

At this level, the sympathetic chains give off numerous nerve bundles which pass in front of the aorta and form a very rich preaortic plexus, in which few nerve cells can be found.

Groups of nerve fibers reach the duodenum and the small intestine along the mesenteric attachment and they encircle these organs, forming a rudimentary myenteric plexus which is still poorly developed on the antimesenteric aspect. Figure 3 is a photomicrograph of a transverse section passing at the level of the small intestine; sympathetic fibers can be seen surrounding the aorta, and from this preaortic plexus they can be followed between the gonads, along the mesentery, and on the mesenteric attachment of the intestine. In the same section, there is an oblique section of the large intestine

with the nerve of Remak cut in two places. A long nerve trunk can be seen running along the vitelline vein, toward the umbilical region.

A higher magnification of the pancreatic innervation is shown in figure 4, where the pancreatic nerve bundles are very small. Coursing between the adrenals, they pass along the coeliac artery and in the dorsal mesentery where they



Figs. 3 and 4 Photomicrographs of transverse sections (by Dr. L. C. Simard) Bielschowsky-Paton impregnation method. Figure 3, near level of umbilical pedicle. Figure 4, at level of pancreas.

reach the pancreas and penetrate into the organ as delicate branches. Some fibers can be followed as far as the mesenteric artery which ingrooves the pancreatic tissue.

From this brief description, it is obvious that the innervation of the anterior intestine and of the pancreas is formed by branches deriving from the sympathetic chains.

It is interesting to note that the innervation of the small intestine is derived from three embryonic segments, as shown by the number of sympathetic ganglia of the prevertebral chains. Although the preaortic plexus is richly developed all along the abdominal region, it does not contribute any fiber to the small intestine, excepting in these three segments. A typically located solar plexus with voluminous ganglia does not exist, but the origin of intestinal and pancreatic nerves is apparently limited to these three pairs of ganglia. This, of course does not eliminate the possible participation of fibers from neighboring ganglia running in the sympathetic chains.

THE POSTERIOR INTESTINE

All along the dorsal aspect of this segment of the digestive tract, the intestinal nerve of Remak can be followed. It gives off on either side a few branches forming a poorly developed nervous plexus in the walls of the hind-gut.

The nerve of Remak originates from the visceral branches of the sacral nerves; these branches form a well-developed preaortic plexus from which numerous cellulo-fibrillar bundles converge toward the dorsal aspect of the intestine; to this plexus the name of Remak's ganglion can be given. The plexus is in direct continuity with the sympathetic chains and with the preaortic plexus of the lower abdominal region.

Cephalically, the nerve of Remak extends as far as the umbilical pedicle. At this level, evident anastomotic branches connect the nerve of Remak with the nervous bundles innervating the anterior intestine; whether these anastomoses represent fibers passing from the coeliac plexus into the nerve of Remak or fibers extending from this nerve into the small intestine branches, cannot be ascertained.

SUMMARY

The esophagus, the proventriculus, and the gizzard have a double innervation deriving from the pneumogastric nerves and from the sympathetic chains.

The duodenum, the small intestine, and the pancreas are exclusively innervated by branches of the preaortic plexus in the coeliac region.

The large intestine is supplied by the intestinal nerve of Remak. In the region of the umbilical pedicle, the nerve of Remak is in direct continuity with the branches innervating the small intestine.

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OBSERVATIONS ON THE FOLLICULAR CYCLE AND ON THE PRESENCE OF THE 'MACROTHYRO- CYTE' IN THE HUMAN THYROID¹

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SEVEN FIGURES

Recent studies on the thyroid gland of the dog have shown that the microscopic structure of this gland is not of a permanent character, but changes gradually so far as the follicular unit is concerned. Both disintegrating and developing follicles are found. These two stages in the history of the follicle, representing the beginning and the end of its existence, are to be distinguished from the period of its complete development and its active function in secretion. In duration, this intermediate stage is the longest, and therefore represents the numerically dominating structure seen in microscopic sections. It requires the examination of series sections to find the small areas containing disintegrating or newly developing follicles. This follicular cycle explains the differences in the structural pictures of the various sections through the thyroid gland. In previous papers ('31 and '32) the writer described such changes and also the existence of a special type of large cell in the thyroid gland of the dog.

Somewhat later (1932), Nonidez published a paper in which he, too, points out the existence of special large cells in the thyroid of the dog. A comparison of his findings with those of the present writer shows their fundamental unity in important respects. But, several months later (August, 1932), he reviewed Zechel's work in a critical paper, in which, in-

¹ Submitted for publication January, 1933.

stead of emphasizing the basic points of agreement, he conveys the impression that the two investigators are in diametric opposition. The chief points of contention can be summarized as follows:

In regard to the large thyroid cells, just indicated, Zechel ('31) described them as 'spherical' and possessing "a homogeneous protoplasm and a large, lightly staining nucleus with a distinct net of chromatin." According to Nonidez ('32 b), "these cells differ from the follicular cells in that they are much larger, contain clear, vesicular nuclei, and eventually leave the follicular wall." The difference in the staining methods employed by the two authors explains why Nonidez found a granular structure of the protoplasm of these cells and Zechel a homogeneous. Zechel used the term 'large thyroid cell' (better 'macrothyrocyte') to designate this cell type, for, at the present state of knowledge concerning it, size is the main feature that distinguishes it from the common thyroid cell. It can be recognized in sections stained with any method, whereas the apparent differences in protoplasmatic structure depend on the technical methods employed. The term 'parafollicular cell' is confusing, since the common thyroid cells located in the follicular wall also leave the follicle and form interfollicular cell groups, just as do the large cells, hence are no less parafollicular.

Respecting this normal phenomenon of follicular destruction, Zechel ('31) remarks "that the follicle is not the only structure of morphological importance in the thyroid gland," and that "in time its cells lose their contiguity and become intermingled with colloid." Nonidez ('32) concurs when he says that "as a result of the continuous segregation of cells from the follicular epithelium loose clusters of parafollicular cells are eventually formed."

Both authors maintain that the 'large cell,' or 'macrothyrocyte,' is in some way concerned with the production of colloid. Zechel: "Because the large cells are so frequently associated with colloid—even with the free colloid located in the interfollicular spaces—and because a small group of

such cells may contain a droplet of colloid in its center, the conclusion is near that they are engaged both in the production of colloid (like the common thyroid cells) and in the new formation of follicles." Nonidez: "The differentiation of parafollicular cells is in some way related to the deposition of colloid within the follicles."

The macrothyrocytes seem to be a characteristic feature of formatively active glands, because they are frequent not only in regenerating glands, as observed by Zechel, but also in young glands, for Nonidez says that "the formation of parafollicular cells is of widespread occurrence during early post-natal life."

Concerning Zechel's conclusions that the 'macrothyrocytes' are associated, 1) with the formation of new follicles; 2) with the production of colloid, and, 3) possibly with the inception of follicular destruction, Nonidez believes that his "studies proved that the role of these elements is very different from that assumed by Zechel," though indirectly he admits the correctness of these conclusions. Regarding the first point, Nonidez says: "Observations on the thyroids of adult dogs, prepared with the method of Cajal, have shown that parafollicular cells are rare in these animals, except in localized areas occupied by small and medium sized follicles (fig. 12). These areas are of wide occurrence in normal thyroids, and judging from the small size of the follicles they contain, they undoubtedly represent foci of differentiation of new follicles." This shows clearly that Nonidez, like Zechel, assumes a connection of the large cells with the formation of new follicles. Although Nonidez here accepts the existence of new follicles, he remarks on the same page that these are not new follicles, but only 'caps' of larger follicles. Why these are not tangential sections, Zechel showed in his first paper ('31 a), and Nonidez ('32) also is opposed to the interpretation of them as 'tangential sections,' for he says: "The view, recently advanced (Wilson, '27), that no interfollicular epithelial cells exist in the thyroid, and that the method of reconstruction with wax plates shows that the cell masses are

really tangential sections of follicles, would not apply to young dogs, for the separation of cells from the epithelium in these animals can no longer be doubted."

As regards the second point made by Zechel, namely, the participation of those cells in the production of colloid, it is interesting to note its corroboration by Nonidez's statement:

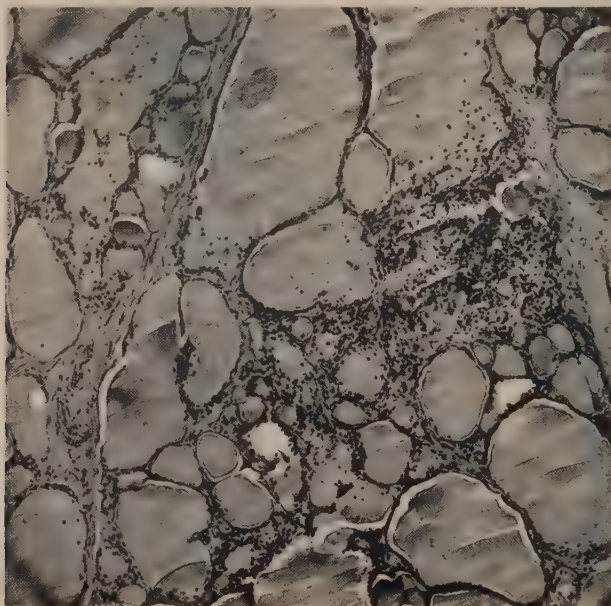


Fig.1 Photomicrograph of a section through the thyroid of a human female, 3 years of age, showing the disintegration of the follicular wall, which has produced an area of free colloid intermingled with the cells that formerly lined the follicles. $\times 101$.

"This strengthens the view, expressed in my former article, that the formation of parafollicular cells is in some way related to active deposition of colloid within the young follicles."

Zechel's third point is also admitted by Nonidez: "It is true that some follicles may be destroyed, as for instance, when numerous parafollicular cells withdraw simultaneously

from the follicular epithelium. Under such conditions, the follicular cells are unable to spread themselves across the gap left by the migrating elements, and the exposed colloid may leave the follicle and enter the interfollicular spaces."

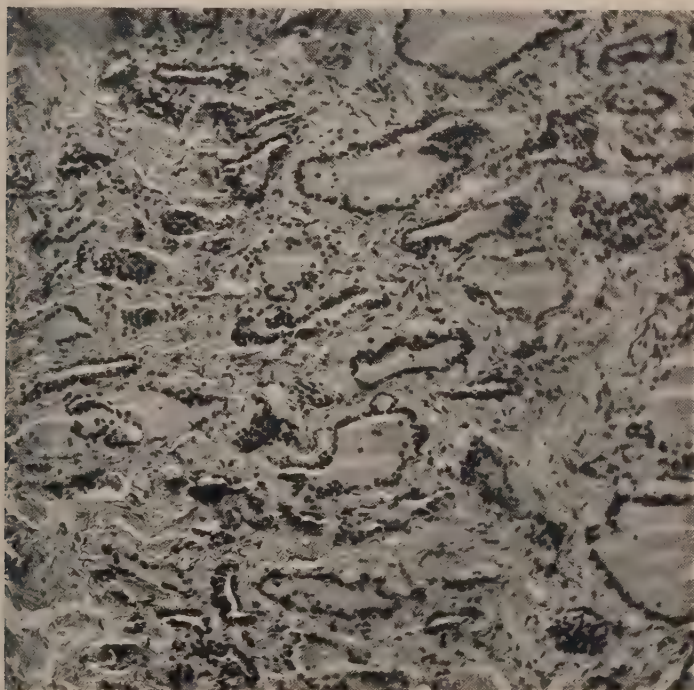


Fig. 2 Photomicrograph of a section through the thyroid of a human male, 24 years of age, illustrating different stages in the collapse of follicles, from one that is still more or less spherical to one that is flattened, and represented by a double row of cells. $\times 163$.

After the foregoing critical comparison of observations made by Nonidez and the writer on the thyroid of the dog, the results of an examination of a series of eighteen normal human thyroids of different age, sex, and race are now presented. The material was kindly made available at autopsies by Dr. Richard H. Jaffe, director of the Pathologic Institute of Cook County Hospital.

The disintegration of follicles of the human thyroid shows differences in appearance. The epithelial lining of adjoining follicles may break down leading to their confluence and that of their colloid content. This process, repeatedly taking

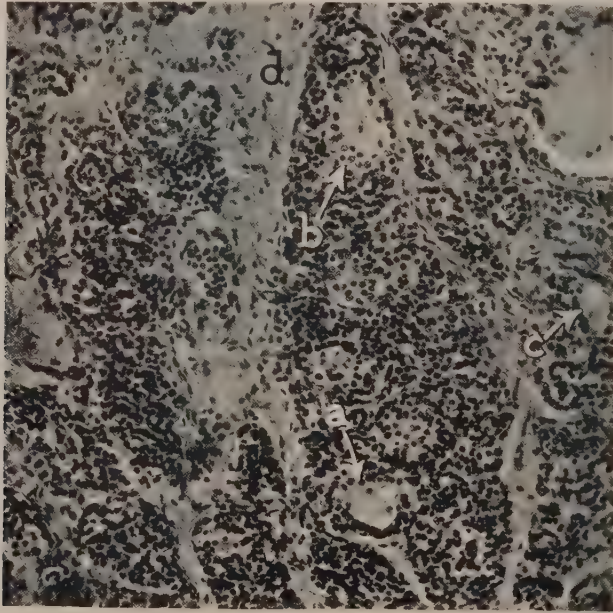


Fig.3 Photomicrograph of a section through the thyroid of a human male, 6 months of age, showing a solid cell mass typical for the juvenile thyroid. $\times 180$. *a*, *b*, and *c* indicate young follicles. In *c* there is only a slightly distinct follicular wall and the colloid seems to be situated freely between the cells, while in *a* and *b* the cells form a definite follicular wall around it. The lower part of follicle *b* and the area to the lower right of the latter *b* contain nuclei typical for the macrothyrocytes. *d* indicates a septum of connective tissue.

place in the same area, may cause the formation of cysts, which perhaps also explains the cysts frequently found in thyroids pathologically changed. The difference between the normal and the pathologic would then only be a quantitative one. On the other hand, two other kinds of follicular disintegration are not characterized by the accumulation of the

existing colloid into one fused mass, but are associated with its disappearance. In the collapse of the follicles either small remnants of colloid are seen in their lumen or the colloid is entirely lacking. Such follicles are represented merely as a potential space between two parallel walls, and finally even this last vestige of a former follicular arrangement disap-

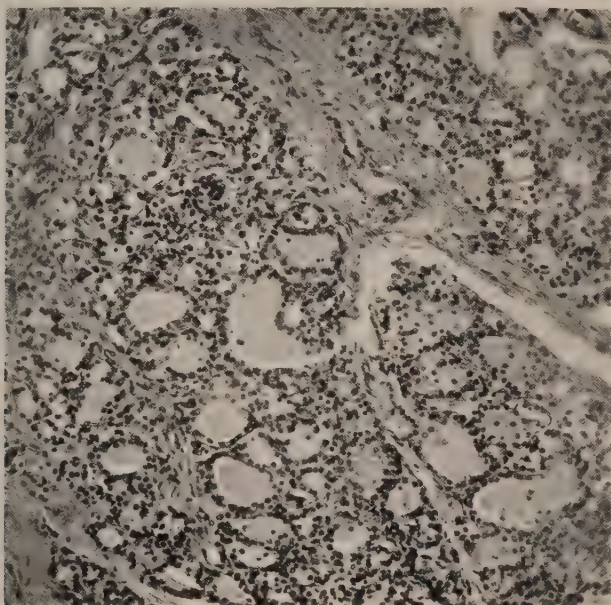


Fig. 4 Photomicrograph of a section through the thyroid of a human male, 6 months of age, showing an area of very young follicles and free embryonic cells that form interfollicular cell groups not arranged as yet into follicular formations. $\times 163$.

pears when the cell rows are broken up into irregular cell masses (fig. 2). This sequence of events is found more frequently in the dog thyroid than in the human. The second type of follicular disintegration, accompanied by the disappearance of colloid, is more common in the human thyroid, and is characterized by areas in which colloid and cells are irregularly intermingled. These two latter types of disinte-

gration seem to be associated in some way with the resorption of colloid, for it is evident that in the disarrangement of the epithelium, a closer contact is established between the vascular channels and the stored colloid.² Can it be that the in-

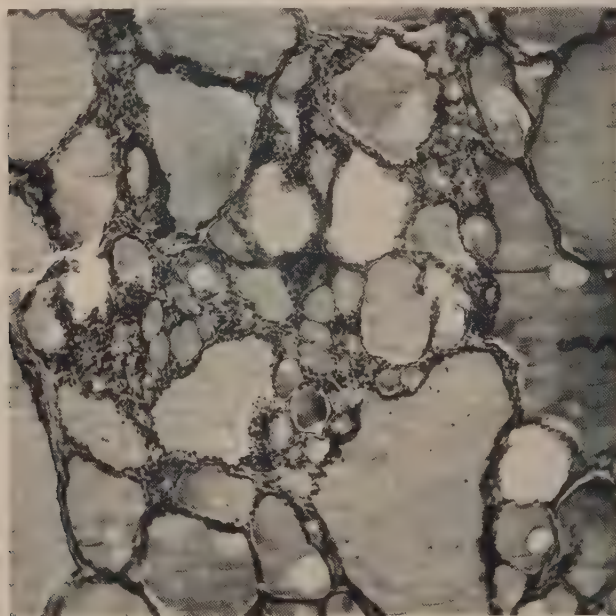


Fig. 5 Photomicrograph of a section through the thyroid of a human female, 20 years of age, showing small follicles that represent reserve tissue among fully developed follicles. $\times 90$.

tactness of the colloid in the first type of disintegration is explained by possible changes in its composition, making it unfit for resorption?

The three types of disintegration described are independent of each other, so that the existence of a follicle comes to an end in either one of these three possible ways, though they

² The term 'stored colloid' is used to indicate the difference between the secretory product of the inner pole of the follicular cell that has been deposited in the cavity of the follicle and that of the outer pole which may enter into the circulation immediately after its formation.

may coexist locally. The result of the last two ways leads to a similar histological picture showing irregular masses of cells, in one case this occurring after the collapse of the follicle; in the other, directly, without such an intermediate stage.

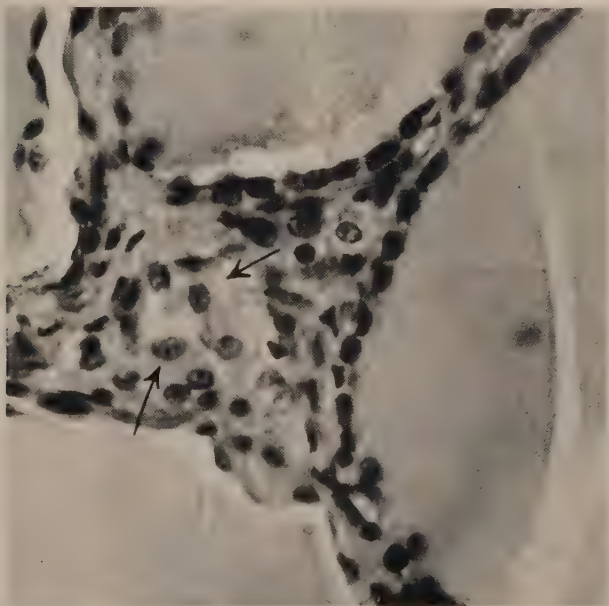


Fig. 6 Photomicrograph of a section through a thyroid of a human male, 70 years of age, showing how the macrothyrocytes form an interfollicular group, and how readily they may be distinguished among the ordinary thyroid cells. $\times 670$.

The cell masses resulting from the disintegration of follicles must be strictly distinguished from embryonic cell masses in the juvenile thyroid that have produced but few if any follicles. Both cell masses, differing in origin and not arranged as follicles, have been termed interfollicular cells. Figures 1, 3, and 4 illustrate their difference. In the first figure the cells shown are derived from disintegrated follicles and are irregularly intermingled with colloid. On the

other hand, the interfollicular cell masses seen in figure 3 are of embryonic origin. Here, the cells are closely packed and do not show any colloid between the cells, as depicted in figure 1. Only three follicles were formed in this area and are indicated with arrows.

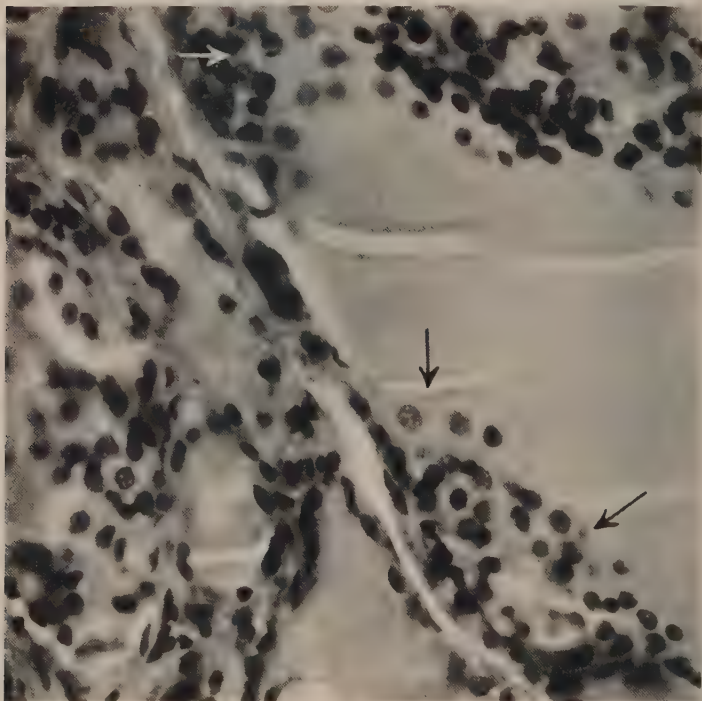


Fig. 7 Photomicrograph of a section through the thyroid of a human male, 70 years of age. $\times 550$. In the lower half of the photograph the arrows indicate macrothyrocytes which form a small part of the follicular wall. Compare the size, nuclei, and chromatic network of the large cells with those of the common thyroid cells.

The continual loss of 'typical' thyroid tissue is accompanied by a continual restoration of such tissue in the formation of new follicles. Young tissue can be found in every thyroid, in the form of irregular cell masses, as in young

children, figure 3, or in the form of cell groups containing small follicles, figure 4, or finally as groups of small follicles in the adult, figure 5. These are the sources from which the regeneration of the thyroid gland takes place.³ The continuous process of destruction and new formation of follicles in the thyroid was termed its 'follicular cycle.'

The large thyroid cells, the 'macrothyrocytes,' described in the dog are also present in man. Figures 6 and 7 picture these cells of the human thyroid. As is evident, they are larger than the common thyroid cells; this is true also of the nuclei which contain a more distinguishable chromatic network. In the macrothyrocyte, the ratio of cytoplasm to nucleus is in favor of the cytoplasm, whereas in the common cell the amount of cytoplasm is comparatively much less. The macrothyrocytes occur most frequently in the interfollicular cell groups, less frequently among the cells lining the follicles, as portrayed in figures 6 and 7.

SUMMARY

1. As in the dog, there is in the human thyroid a follicular cycle consisting of destruction and new formation of follicles.

2. There are interfollicular cell groups composed either of embryonic cells or cells derived from the disintegration of follicles.

3. In addition to the ordinary cells, the typical large cells, the macrothyrocytes, recently described in the dog, occur also in the human thyroid.

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³ See G. Zechel, Experimental regeneration of the thyroid gland in the dog. *Surgery, Gynecology and Obstetrics*, 1931.

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THE 'PARENCHYMATOUS' CELLS OF BABER,
THE 'PROTOPLASMAREICHEN ZELLEN' OF
HUERTHLE, AND THE 'PARAFOLLICU-
LAR' CELLS OF THE MAMMALIAN
THYROID

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ONE PLATE (FIVE FIGURES)

In two former articles (Nonidez, '32 a, b) the transformation and separation of cells from the follicular epithelium of the thyroid of the dog and cat has been reported. These observations have been extended to the rabbit by Miss N. Raymond ('32). The elements that become separated from the walls of the follicles were termed 'parafollicular' cells, since they lie in close proximity to the follicles.

In my first contribution it was stated that the parafollicular cells of the dog thyroid must have been seen by other investigators, since they are large, and can be identified in sections stained with the routine methods. At the time I was not aware of two articles by Zechel ('31 a, b) in which large interfollicular cells derived from the follicular epithelium are described. A third article by this author appeared simultaneously with my paper (January, '32). The findings of Zechel have been discussed elsewhere ('32 b).

A search of the older literature on the thyroid gland has revealed that the elements termed by me 'parafollicular' cells were first described by Baber (1876, 1881) under the name of 'parenchymatous' cells. According to this investigator, these cells arise, or are first located, in the interfollicular spaces and migrate toward the follicles, which they eventually enter, but he was unable to state what part they take in

the formation of their contents. In his second article, Baber mentioned the presence of similar cells in the thyroid of the cat, rabbit, and pigeon. The findings of Baber were confirmed in part by Huerthle (1894), who termed the elements under consideration 'grosse' or 'protoplasmareichen Zellen.' However, Huerthle believed that these cells do not enter the follicles, but that they are gradually incorporated into their walls, contributing to their growth. Clear examples of large cells embedded in the follicular epithelium can be seen in Huerthle's figure 2, while a cluster of these cells is represented in the lower half of his figure 4.¹ He also stated that the large cells give rise to new follicles.²

The large cells described by Baber and by Huerthle were also observed in the dog thyroid by Takagi ('22), who has described some of their cytological characteristics in sections stained for mitochondria. According to this investigator, "a minute examination of these elements shows that they greatly differ from the follicular epithelium." In very young puppies (3 to 5 days after birth) the cells under consideration contain very numerous, small plastosomes (mitochondria). At this stage neither granules nor vacuoles occur in their cytoplasm. He emphasizes the fact that during this phase the interfollicular cell "cannot be classed with the follicular epithelium cells whatever its position may be," that is, even in those instances in which it appears embedded in the wall of a follicle.

In the glands of older puppies Takagi found that the interfollicular cells are larger, and their mitochondria steadily

¹ Both figures were copied from sections of thyroids of dogs preserved with sublimate-acetic mixture, and stained with the method of Biondi. In addition to the large cells, Huerthle described smaller elements, called by him 'protoplasmarme Zellen.' These cells are similar to the normal follicular cells, but they show "eine deutlich ausgesprochene Granulierung" in their cytoplasm. In some instances they are larger and "nähert sich dem der protoplasmareichen Zellen" (p. 39).

Lobenhoffer ('09) expressed the opinion that, since the small cells have granules in their cytoplasm, they might produce some substance acting as a stimulus for the formation of new follicles.

² "Die Entstehung (of new follicles) geht entweder von protoplasmareichen Zellen aus oder diese Zellen finden sich wenigstens in der Umgebung" (p. 40).

decrease in numbers until they are represented by a few rod-shaped corpuscles. At the same time granules develop, and numerous vacuoles appear in the cytoplasm. The granules, judging from Takagi's figures, are fewer in number and coarser than in the silver preparations; furthermore, they show an outer, deeply stained zone, and a clear core in sections stained with iron hematoxylin. The large cells, on the other hand, are much more numerous than in the younger puppies. In 2-month-old puppies, according to the author mentioned, cells filled with mitochondria are found only here and there, while the elements containing granules, vacuoles, and few mitochondria prevail.

From his observations Takagi concludes that the large interfollicular cells are of glandular nature, and that the secretion they produce is contained within the numerous vacuoles that occupy their cytoplasm. As to the path of discharge of the secretion of these cells he states that "it would appear that it finds a reservoir for a certain period in the interfollicular space, though we are left in the dark as to whether the secretion so stored up there is thenceforward absorbed directly in the interstitial lymphatic space or whether after being discharged into the follicular lumen it constitutes an element of the colloid mass." However, he states, in agreement with Huerthle's view, that the large interfollicular cells often appear wedged among the follicular elements, and "from this fact it will follow that undoubtedly at least a part of the secretion is drained out into the follicular lumen" (p. 92).

It is interesting to note that the two different aspects of the interfollicular cells noticed by Takagi with mitochondrial methods correspond very closely to two different aspects of the parafollicular cells in silver impregnations. I have already indicated ('32a) that in newborn or very young puppies the parafollicular cells are large, clear elements with relatively few, faintly argyrophilic granules (fig. 5). According to the findings of Takagi, this is the stage when the small mitochondria are exceedingly numerous. On the other

hand, in older puppies and in the adult, the parafollicular cells are loaded with intensely argyrophilic granules (figs. 1 to 4). This aspect corresponds to the stage in which the mitochondria methods reveal few mitochondria and the presence of annular granules, already mentioned. The relative absence of mitochondria is explained by the fact that the cytoplasm is now occupied by granules that Takagi was unable to stain with his technique. This is further proof that the argyrophilic granulations are not mitochondria, but that they are constituted by a specific substance which, in the dog at least, shows marked affinity for the silver. As to the vacuoles noticed by the Japanese investigator, they occur, for the most part, in cells in which dissolution of the granules in the cytoplasm is already well advanced. Such vacuoles, as stressed by Takagi, do not occur in the follicular cells; instead there are large, coarse granules which stain deeply with iron hematoxylin.

The point of chief interest in the observations of Takagi is that, although he failed to give a complete account of the origin of the large interfollicular cells, he realized that these elements differ considerably from the ordinary follicular cells, and that their nature is undoubtedly secretory.

The direction of the migration of the parafollicular cells is a matter of no small importance, for if they join the follicles from the outside they may be instrumental in the growth of their walls—a role attributed to them by Huerthle—or in the production of a secretion joining the colloid, as hinted by Takagi. On the other hand, if the cells under consideration leave the follicular epithelium to enter the interfollicular spaces, as claimed independently by Zechel and by the writer, their function must be different.

Evidence favoring the view that the parafollicular cells do not join the epithelium lining the follicles, but that they arise from elements scattered among the ordinary follicular cells, is found in sections prepared with the reduced silver nitrate method. I have already indicated ('32 a) that when only two or three parafollicular cells leave the follicular epithelium in

a given area of the follicle wall the adjoining follicular cells spread over the exposed colloid and fill the gap left by the migrating elements. It must be admitted, however, that the histological picture in these cases could be interpreted in a different way. According to the views of Huerthle and Takagi, the thin area of follicular wall interposed between the parafollicular cells and the colloid would result from pressure exerted by the large elements as they push their way to reach the colloid. In a similar manner, the elongation of the follicular cells in contact with the parafollicular elements might be accounted for as the result of their forcible separation or compression by the larger cells that are being incorporated into the follicular wall. More convincing evidence on the separation of the parafollicular cells from the follicular epithelium is, therefore, necessary to settle the point.

If the view that the parafollicular cells move away from the walls of the follicles is true, instances should be found in which simultaneous separation of a number of these cells from a restricted area of a follicle would lead to its rupture. Figures 1 and 2 represent three stages in this process. In order to meet the possible criticism that rupture of the follicles might be due to traumatic agencies (i.e., to handling of the gland) I have selected only those follicles occurring in the center of the thyroid.

Figure 1 shows two follicles separated by connective tissue. In the follicle to the right of the figure several parafollicular cells occupy an area of the wall, but their separation from the follicle is not yet initiated. The follicle to the left shows a more advanced stage. The parafollicular cells are rounder, they are beginning to lose contact with each other, and as a whole are out of alignment. Between the migrating elements and the colloid a space is noticeable. In this, as in the other figures, the follicular cells are in contact and aligned as in normal follicles. This indicates that the follicles represented were not undergoing disintegration at the time of fixation. On the other hand, it must be stated that in normal glands of puppies disintegration or rupture of the follicles rarely

takes place, but in older dogs follicular disintegration may be present in many areas of the gland.

Figure 2 shows an exceptional case of rupture of the follicle wall in the area formerly occupied by several parafollicular cells. The latter are no longer in contact with each other, and the more fluid part of the colloid seems about to escape from the follicular cavity. An elongated follicular cell (*e*) is seen wedged between two of the parafollicular elements. In terms of the hypothesis of Huerthle and Takagi, the figure would be interpreted as showing several parafollicular cells about to be incorporated into the follicular wall. Yet, it would be necessary to admit that the latter has previously been dissolved or reabsorbed in the area facing the parafollicular elements, even though no remnants of cells or nuclei occur in this area.

Similar difficulties are encountered in the interpretation of follicles in which a part of the wall facing groups of parafollicular cells is separated from the latter by a wide space and appears considerably flattened (figs. 3 and 4). It may be argued that the intervening space in this case is due to shrinkage during the preparation of the sections. That there is some shrinkage in both the follicles and the parafollicular cells³ cannot be doubted. Nevertheless, the wide spaces cannot be regarded entirely as artifacts, for, if they were, one should not expect to find parafollicular cells in contact with the follicular wall in the immediate vicinity or in another area of the same follicle (fig. 4, cells to the right). The flattening of the follicular cells in these cases is significant, since it may not be due to pressure exerted by those parafollicular elements about to be incorporated into the follicu-

³ Baber noticed spaces between these cells when they occur in groups, and he attributed their presence to shrinkage. The occurrence of wide intercellular spaces was confirmed by Huerthle. Takagi does not believe that the large cells undergo shrinkage more readily than the follicular elements. I am of the same opinion, for no intercellular spaces are noticeable between those parafollicular cells still embedded in the follicular epithelium (fig. 1, follicle to the right). The spaces between the parafollicular cells in the clusters may result from the release of a fluid secretion produced through dissolution of the granules in their cytoplasm (Nonidez, '32 a).

lar wall, but rather to the fact that follicular cells have spread themselves over the colloid to close the gap left by the migrating parafollicular cells.

As already stated, Huerthle believed that the large interfollicular cells are instrumental in the formation of new follicles, and a detailed description of the processes involved is given by this investigator. The same view has been expressed recently by Zechel ('32). This aspect of the problem has been already discussed ('32 b). The fact that no parafollicular cells exist in many of the follicles being formed in the thyroid of puppies shortly after birth would seem to indicate that their participation in this process is not essential.

The preceding considerations are based on a study of the thyroid of the dog. In the rabbit the gradual increase in the numbers of parafollicular cells as the animal becomes older is still more manifest (Raymond, '32). The increase in the interfollicular epithelium has not escaped other observers. Thus, Biedl ('22) states that "bei jugendlichen Individuen und manchen Tierarten, wie z.B. beim Kaninchen, trifft man die Follikel wenigen zahlreich und verhältnissmässig viel interfollikuläres Epithelgewebe" (p. 160). Due to the presence of denser fibrous tissue in the stroma of the gland of the rabbit, the migration of the parafollicular cells is not so marked as in the dog, and these elements may form an uneven layer around the periphery of the follicles, closely resembling the arrangement of the granular peripheral cells described by Bensley ('14) in the thyroid of the opossum.

In concluding I wish to say that while credit is due Baber and Huerthle for their accurate descriptions of the large interfollicular (parafollicular) cells, their interpretation of the origin and fate of these elements receives little support in the facts disclosed by a technique more specific than the staining methods used by these early investigators.

CONCLUSIONS

1. The elements termed by the author parafollicular cells were discovered in the thyroid of the dog by Baber, and their presence was confirmed later by Huerthle. They have been studied more recently by Takagi. According to these investigators, the large interfollicular (parafollicular) cells join the follicles, which they enter (Baber), or they contribute to the growth of the follicular walls and formation of new follicles (Huerthle). According to Takagi, they are glandular cells that may pour their secretion into the cavity of the follicles.

2. The views of the authors mentioned above are discussed, and additional evidence is presented pointing to the separation of the parafollicular cells from the follicular epithelium.

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PLATE

PLATE 1

EXPLANATION OF FIGURES

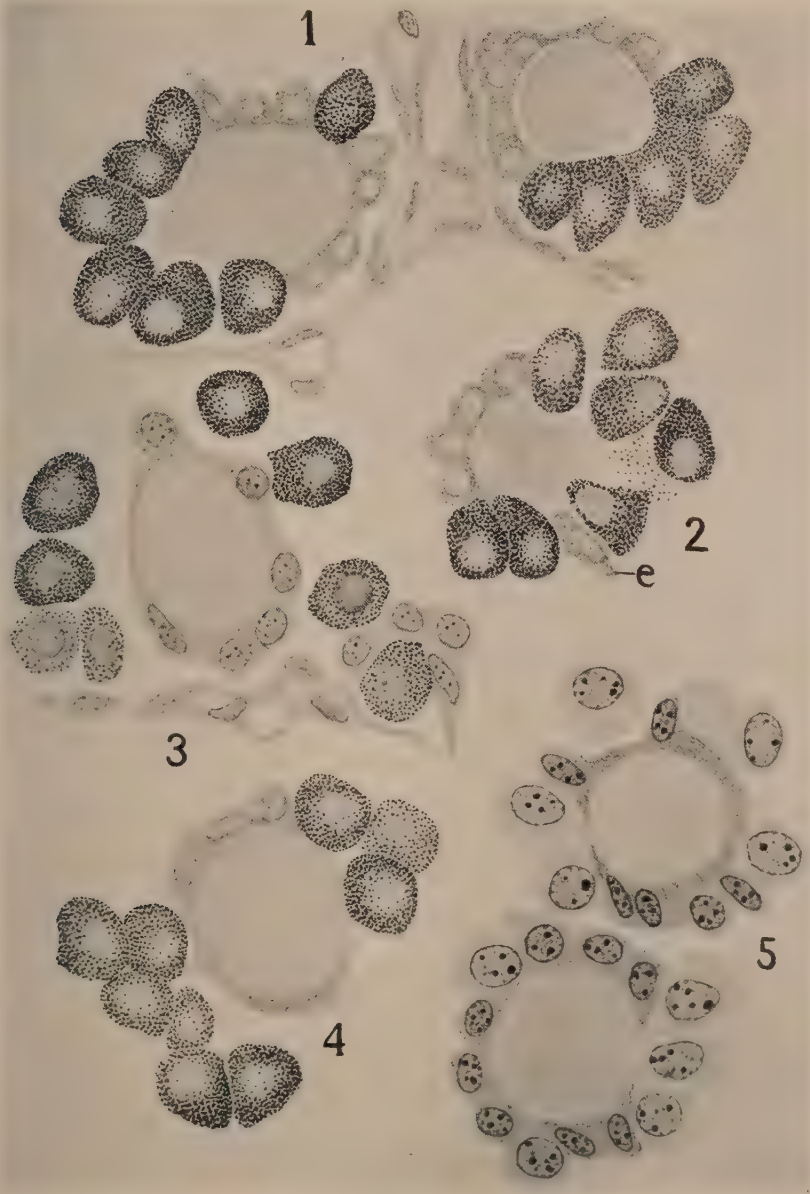
All figures were copied from sections of thyroids prepared with the reduced silver nitrate method of Cajal, and were drawn at the same magnification.

1 Two stages in the separation of several parafollicular cells from the follicular epithelium. Five-week-old puppy. Alcohol-ammonia fixation.

2 Rupture of a follicle after the separation of several parafollicular cells. *e*, elongated follicular cell. Same puppy as in preceding figure.

3 and 4 Follicles with flattened areas in their epithelium after separation of several parafollicular cells. Adult dog. Alcohol-chloral hydrate fixation.

5 Two follicles of the thyroid of a 2-day-old puppy, showing thin walls (upper follicle) and absence of strongly argyrophilic granules in the cytoplasm of the parafollicular cells. Alcohol-chloral hydrate fixation.



VITAL AND SUPRAVITAL STAINING OF ERYTHROCYTES

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Present opinion is far from unanimous as to the nature and significance of the granules demonstrable in fresh vertebrate erythrocytes following exposure to such vital dyes as methylene blue, neutral red, and brilliant cresyl blue. Many investigators believe that the staining reaction is an agonal process which is not characteristic of the normal living red blood cell.

Nittis ('30), one of the more recent investigators of this subject, working with the blood of the frog and pigeon, asserts that proof is lacking that red blood cells, conclusively alive, can be vitally or supravitaly stained and still continue to live. Such proof, Nittis points out, is difficult to obtain in that we lack specific criteria to demonstrate whether an erythrocyte is alive or dead at any given time when it is outside the circulation.

His conclusions are based on a series of experiments with brilliant cresyl blue as the chief vital dye. The dye was applied intravenously in a normal saline solution. When the amounts introduced were sufficiently small that the animals were not injured, no staining of the erythrocytes in the circulation was obtained; but when so large an amount of the solution was injected that the animals died, the erythrocytes were 'vitally' stained.

Nittis also found that erythrocytes which had been removed from the circulation and kept for many days would take up in a similar manner dyes applied by the supravital method. He believes, therefore, that viability of the cell is not neces-

sary for 'supravital' staining, and that the only essential factors are that the cells must float and must float in their natural medium, the blood serum.

Nittis argues that the objection that the stain might be modified in the circulation of the living animal, thus losing its staining properties, is discredited by the fact that when a large amount of the solution is injected, and the animal dies, the erythrocytes in the blood vessels are stained. From these data he concludes that it cannot be demonstrated that erythrocytes can continue to be alive after staining. He suggests that the cells could be in a living condition at least at the moment immediately preceding the entrance of the dye, but points out that even this is not a required condition, since dead cells suspended in serum stain in a similar manner.

Two objections may be raised to Nittis' conclusions. Practically all his observations were based on experiments with brilliant cresyl blue. This dye is reduced rather readily in the living organism and usually must be present in considerable excess if any visible staining reaction is to be obtained. Moreover, the fact that red blood cells could be 'vitaly' stained while in a state excluding any possibility of viability does not prove that all cells which stain in a similar manner are necessarily not living.

In order to test the validity of these objections, a series of experiments, similar to those of Nittis on the frog and pigeon, were carried out on *Necturus*. Both brilliant cresyl blue and neutral red were used. The latter is not readily reduced.

MATERIALS AND METHODS

Adult *Necturus* were used and the dyes, made up as saturated solutions either in 0.85 per cent sodium chloride or in cold-blooded Ringer's, were introduced intraperitoneally. The amount injected at one time varied from 0.5 to 1.0 cc. In some instances daily injections were made; in other cases animals received the dye on alternate days. The total amount of the saturated solution of either dye given to any animal seldom exceeded 4.0 cc. With this dosage the dyes were not appreciably toxic and no animals died from the treatment.

Neutral red is not destroyed or rapidly eliminated, and with repeated injections the animals assumed a deep red color. When the animals were killed and dissected many evidences of neutral red storage were found in various organs and tissues, and some unabsorbed solution was usually present in the peritoneal cavity. With brilliant cresyl blue, on the other hand, daily doses of 0.5 cc. were insufficient to produce any external evidences of an increasing accumulation of the dye and all the tissues, organs, and body fluids were found to be normal in color. However, when the dosage was increased to 1.0 cc. daily, evidences of dye accumulation in the blood stream were obtained after the third injection.

The progress of the vital staining of the erythrocytes in the circulation was checked by examination of fresh blood drawn from one of the branchial vessels. Dry films stained by Wright's method were also prepared, and at the close of the experiments with neutral red the more vascular tissues, liver and mesonephros, were fixed and prepared according to the method of Forkner ('30) for the preservation of neutral red in tissues stained supravitaly.

OBSERVATIONS

The typical mature erythrocyte of *Necturus* is characterized by the presence of two clusters of small granules suspended in its cytoplasm in the vicinity of the poles of the nucleus. The granules give a basophilic reaction with Wright's stain, are blackened with osmic acid and silver, are stained by iron hematoxylin after fixation with Helly's fluid, and also may be stained supravitaly with the common basic vital dyes (Dawson, '28). Under unfavorable conditions in supravital preparations, such as an excess of dye, high temperature, or too brilliant illumination, a secondary staining reaction is obtained. An increase in the size and number of the granules of the bipolar clusters usually occurs first; later, other scattered granules appear in variable numbers throughout the cytoplasm. The secondary granules resemble in every respect the primary or preformed granules of the erythrocyte.

The presence of color in these definitely localized primary granules would, accordingly, furnish adequate evidence of the penetration of the dye into the erythrocytes, and the appearance of secondary granules would furnish evidence of some degree of injury produced by the dye's reaching the blood stream from the peritoneal cavity. Furthermore, the death of any considerable number of erythrocytes in the circulation of *Necturus* is rapidly followed by intravascular phagocytosis of the dead cells (Dawson, '30).

a. Neutral red

The bipolar clusters of granules were distinctly colored in the erythrocytes of the blood stream after an animal had received three, successive, daily, intraperitoneal injections (0.5 cc.) of neutral red. Determination of the presence of the dye in the blood cells was made by examination of freshly drawn blood. With greater doses a variable amount of secondary reaction to the dye, i.e., induction of granules, was obtained, but prolonged injections failed to produce any patterns of reticulation. Moreover, the neutral red is not readily lost from the erythrocytes; an examination of fresh blood 15 days after the final injection revealed stained granules in practically all the cells. There was no evidence of any destruction of erythrocytes, and no intravascular phagocytosis was observed. The neutral red was also readily demonstrated in fixed erythrocytes of both liver and mesonephros which had been prepared by Forkner's method. Animals kept under observation for 3 weeks following the experiments did not develop any appreciable anemia, and it was concluded that the penetration of the dye had not resulted in the death of many erythrocytes.

b. Brilliant cresyl blue

The results with brilliant cresyl blue were rather less satisfactory than those with neutral red. Cresyl blue is reduced rather readily and as many as eight successive daily injec-

tions of 0.5 cc. each failed to produce any visible coloration within the erythrocytes. When, however, the dosage was increased to 1 cc. daily, positive results were obtained after three injections.

The primary granules were sharply stained in practically all the erythrocytes, and in many instances a high degree of induction of secondary granules was observed. In addition, many examples of intravascular phagocytosis of erythrocytes were noted, indicating that the dosage employed was toxic to the erythrocytes, although the animal gave no external evidences of injury. Probably an intermediate dosage could be found which would be too large for all the dye to be reduced yet not great enough to be toxic to the erythrocytes. No attempt was made to determine such a dosage.

DISCUSSION

These experiments are interpreted as presenting indisputable evidence that the nucleated erythrocytes of *Necturus* may be vitally stained with either neutral red or brilliant cresyl blue and still continue to live. It also appears that the formation of secondary granules within the cell is not acceptable evidence of its death. Reticular patterns were not produced and staining of the nuclei was not obtained by applying the dye vitally.

Vital staining of the granules within the erythrocytes does not indicate the death of the cells, since animals in which staining was known to have occurred continued to live and later did not present any evidence of a massive destruction of the erythrocytes. Nittis' failure to obtain staining following introduction of the dye into the living animal must be explained by the fact that brilliant cresyl blue is reduced by the living organism. Chambers, Pollack, and Cohen ('29) found that this dye was reduced in 2 to 5 seconds after injection into the cytoplasm of sanddollar and starfish eggs. They also reported this dye as quite toxic. These properties were likewise exhibited when the dye was injected into *Necturus*.

It appears impossible to determine how much induction of new granules is compatible with erythrocyte survival, but examination of blood at intervals after induction was observed in the erythrocytes suggests that the majority of the cells recover from the effects of the dye. The observations of Kedrowsky ('31), using rongalit white on the erythrocytes of fish embryos (*Carassius*), also indicate that vital staining with neoformation of granules is not incompatible with the survival of these cells.

Nittis' observation that erythrocytes which are dead may accept a vital dye when applied by the supravital method was confirmed, but it is illogical to argue from this finding that all cells which take up the dye similarly must be dead. The nucleated erythrocytes appear to behave as do other less specialized cells of the body. They store dyes in preformed granules of their cytoplasm, and when the dye is present in excess, new bodies may appear which also accumulate dye ('Krinome' of Chlopin, '27). Both these reactions may take place within the living cell. Preformed bodies may also be stained in cells which are no longer alive.

The use of the word 'vital' and its derivatives in connection with a staining process or with reference to stainable elements or structures of erythrocytes is not inaccurate and misleading, as inferred by Nittis from his experiments. On the other hand, it is true that dead cells may take up the dye in a similar fashion when they are suspended in their own plasma; and Nittis' proposed term 'hygrochorosis,' if used, should be limited to this latter phenomenon.

SUMMARY

Granules of the cytoplasm of the erythrocytes of *Necturus* may be stained vitally by neutral red and brilliant cresyl blue injected intraperitoneally. Since the animals survive following observed vital staining of the erythrocytes, and since no massive destruction of these cells follows the treatment, it is concluded that vital staining is not necessarily associated with the death of these cells, contrary to the conclusion of Nittis ('30).

When used in small amounts, brilliant cresyl blue is reduced and therefore fails to give visible coloration. Larger doses are not entirely reduced and staining of the erythrocytes is obtained. Heavy dosage with either dye may result in the neoformation of granules in the red blood cells. Of the two dyes, brilliant cresyl blue proved more toxic than neutral red; and with heavy dosage of the former, intravascular phagocytosis of erythrocytes was observed. It appears that a moderate induction of secondary granules is compatible with survival of the erythrocytes. Patterns of reticulation were not produced in living erythrocytes by injection of dyes in sublethal doses.

Dead erythrocytes suspended in their own plasma may be characteristically stained by the supravital method. This need not imply, however, that all cells which stain in the same fashion are dead.

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THE ARCHITECTURE OF THE PECTORAL APPENDAGE OF THE CODFISH

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FIVE FIGURES

The writer has undertaken a thorough investigation of the shoulder architecture in mammals, particularly from a neuro-myological viewpoint. In this connection a necessary step has been the examination of conditions in fishes, and in a recent paper (Howell, '33) was discussed the neuro-muscular features of this part of the dogfish (*Acanthias*). The anterior limb of tetrapods could never have passed through the stage now encountered in this elasmobranch, for the reason that its skeletal components are too dissilimar, and because the myomeres contributing to the fin musculature are entirely too numerous. This animal is capable, nevertheless, of adding considerably to our knowledge of limb morphogenesis, for its 'brachial plexus' is uncomplicated by any but the simplest sort of anastomosis. Hence the innervation of the fin muscles indicates with clarity the exact derivation of each muscular division. Furthermore, the neuro-muscular arrangement of the fin clearly indicates that the initiation of the fin musculature was into a protractor and a retractor series, rather than first into a dorsal extensor and a ventral flexor group.

It is, of course, beyond question that even the most generalized of the teleosts are highly specialized vertebrates, far off the direct pathway that led to tetrapods. But some of their features, although showing elaboration of detail, follow a basic ground plan. A great many teleosts have pectoral fins modified for particular functions; but scrutiny of the more

generalized sorts, with simple fin action, will repay the morphologist. The difficulty is that some of the details, apparently simple, may have become secondarily so. Thus it is likely that the endoskeletal features of the teleost fin proper have been sharply reduced from a more elaborate ancestral plan, and the innervation of the intrinsic muscles indicates that these have been redistributed from a more complex pattern. Nevertheless the myology is of interest because in some forms the fin muscles now occur in an ideally simplified arrangement. It must be understood that there are very diverse uses to which the pectoral fins of fishes can be put, and these members vary much in accordance with the character of their habitual use. The most simple sort is that merely of prime movement and recovery, and one of the most frequently encountered instances of this is the one involving protraction (most often unilateral) of a fin to act as a brake, in which case the fin adopts the vertical plane.

After dissecting the fins of a considerable number of fishes, it is felt that the Gadidae may be selected as presenting in most symmetrical form a general plan of fin architecture. This genus exhibits an essential arrangement that is shared by perhaps the majority of teleosts with features fairly unspecialized, at least of the type lacking a mesocoracoid, while at the same time accentuating certain trends suggested in many groups. With this in view, several cod (*Gadus morhua*), both fresh and preserved, were dissected; and the nerves of living fish, anaesthetized, were stimulated by the faradic current.

Preaxial and postaxial are terms used to designate the borders of the fin that would be, respectively, anterior and posterior were this member still primitively horizontal. The morphological flexors of the fin have now become abductors and the former extensors the present adductors, and they are so termed.

SKELETON

Of dermal elements associated with the so-called clavicular girdle of the cod there is a posttemporal articulating with the skull and with the supracleithrum below, while the latter in turn articulates with a large, ventral, cleithrum, as shown in the figures. In Teleostei the original clavicle has disappeared, as long since pointed out by Gegenbaur. The cartilaginous bones are located partly medial to the dermal girdle and consist of a scapula and a more inferior coracoid (there is no mesocoracoid in this group). Between the latter bones and the fin base there is a series of four small basalia, and medial to all a long, slender postclavicle, so-called, that resembles a first rib in that it is interposed between the body muscles proper and the hypobranchial musculature.

The dermal fin rays, or dermatrichia, in the cod as in all bony fishes dissected, occur in two parallel layers. The base of each of these overlaps that of its neighbor and ends in a projection, of different shape on the two sides of the fin (figs. 4 and 5), those from the medial aspect being particularly variable. The bases of the medial layer of fin rays are located farther proximal than in the case of the lateral layer, and in different teleosts there is much variation in the finer details. The fact that the fin rays occur in two layers in these fish, as is not the case in selachians, has not received the anatomical attention which it merits.

MUSCLES

The nerves show that the preaxial border of the pectoral fin has been rotated upward. Accordingly, the muscles that were formerly dorsal extensors have become adductors of the fin, and the former volar flexors have become a protractor or abductor group. Each of these groups in teleosts is basically composed of two well-differentiated muscles, and each inserts upon the entire breadth of the fin base. In the cod, the dorsal, more superficial division of the abductors arises mostly from the medial surface of the cleithrum, few if any fibers coming from the scapula, and inserts upon the bases of

the lateral layer of fin rays. This may be termed 'm. abductor dorsalis.' It covers the insertional end of the deeper m. abductor ventralis which arises partly from the medial surface of the cleithrum and also from the lateral surface of the coracoid. The fibers from both of these origins insert upon each side of an aponeurosis which is attached, with deeper muscle fibers, upon the bases of the lateral fin rays.

Frequently in teleosts there is a third, minor muscle division of either the abductor or adductor groups, or both, as mentioned by Shann ('20), rarely of large size except in specialized sorts. There is one such muscle in each group in the cod, the more lateral of which may be termed m. abductor accessorius. Its full width is indicated in figure 1, and it arises from the scapula and inserts upon the preaxial fin base.

These three muscles are innervated ostensibly by branches of the diazonal nerve, derived from sp.nn. 1, 2, and 3.

The more superficial of the muscles upon the medial (originally extensor) side of the fin may be termed m. adductor dorsalis. It arises from the dorsal part of the medial cleithrum and the medial scapula, and is inserted upon the entire width of the fin base, but slightly farther distalward than in the case of the abductor. The deeper component, the m. adductor ventralis arises mostly from the anterior border of the cleithrum, from a tendinous arch (fig. 3) stretching over the ventral part of the cleithrum, and slightly from the coracoid. It inserts upon the fin base deep (lateral) to the more superficial adductor. There is still a third element of this group, small and poorly defined. It arises from the dorsal part of the medial coracoid and slightly from the cleithrum, with insertion to the medial base of the preaxial border of the fin. It may be termed m. adductor accessorius.

The innervation of the fin adductors, ostensibly by branches of the diazonal nerve, presents some puzzling features as discussed shortly.

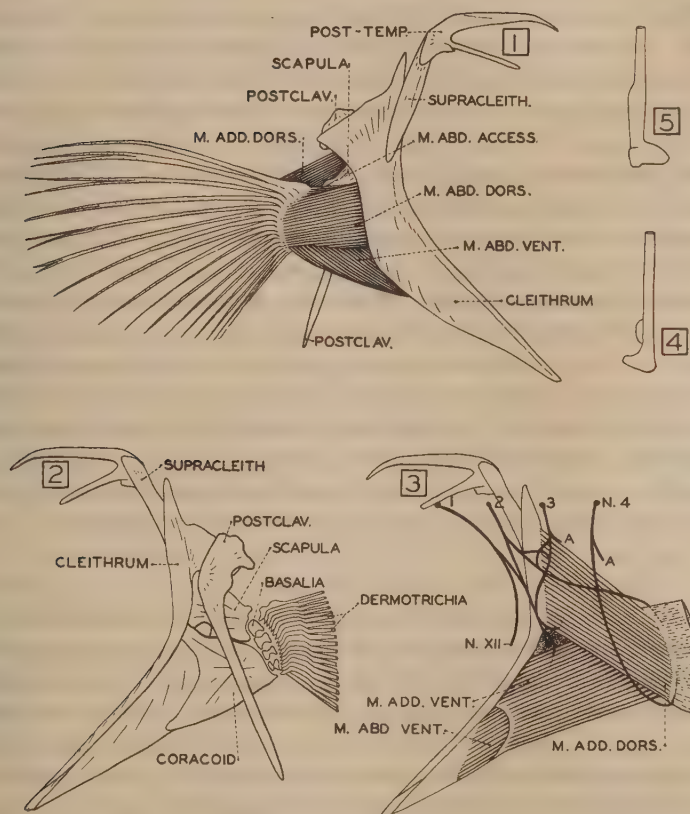


Fig. 1 Lateral view of the right clavicular girdle, pectoral fin, and its intrinsic musculature in the cod.

Fig. 2 Medial view of the right clavicular and scapular girdles of the cod.

Fig. 3 Medial view of intrinsic pectoral musculature in the cod, with distribution of the brachial nerves; postclavicle removed. AA, nerve branches to myomeres of trunk.

Fig. 4 Lateral view of a dermatrichium from the lateral layer of the right pectoral fin. $\times 2$.

Fig. 5 Medial view of a dermatrichium from the medial layers of the right pectoral fin. $\times 2$.

NERVES

The plan of the brachial plexus is indicated in figure 3 and a formal description seems superfluous. The number of neuromeres contributing to the hypobranchial nerve was not investigated. It is seen that the diazonal nerve, piercing the coracoid foramen and supplying the abductor musculature, also sends twigs to both adductors of the fin. The first three brachial nerves anastomose to contribute to these branches. So far there is nothing noteworthy. But branches of the second and third nerves join to send a nerve to the preaxial (dorsal) border of the fin, where it enters between the two layers of fin rays and passes at a right angle to the latter. The fourth nerve has a precisely similar course from the opposite side, entering the fin base upon the postaxial (inferior) border. In addition, it should be mentioned that the first three brachial nerves at least, and apparently the fourth as well, send smaller branches, almost certainly sensory, over the medial surface of the adductor muscles.

DISCUSSION

The brachial plexus of teleosts is now too complicated to indicate precisely from which somites the fin musculature is derived, and the effect of faradic stimulation of the brachial nerves is of too generalized a character to be very helpful. All that is indicated is that the preaxial muscles have been elaborated from somites that were situated more anteriorly than those which contributed to the postaxial muscles, bearing out the contention formerly advanced (Howell, '33) that the original arrangement of the fin musculature was into protractor and retractor groups. This arrangement, however, is best adapted to bottom-living forms, and when the ancestors of the bony fishes took to a free-swimming existence the elaboration was undoubtedly into dorsal or extensor, and ventral or flexor, groups of fin muscles, changed by the rotation of a fin used for unilateral braking into, respectively, adductor and abductor groups. But movement in two directions is not enough for a many-rayed fin, for there must be spreading

and contraction of the fin. In consequence, there has differentiated a more dorsal muscle partially superficial to a more ventral division in both abductor and adductor series. This is the most simple, effective arrangement for manipulating a paired fin of the sort characteristic of the generalized teleost stage.

It will be seen that if there be rotation of the fin plane there must be a compensating shift in the origin of the controlling musculature. Hence one must infer that the origin of the more superficial of the abductors migrated upward along the cleithrum from a position near the coracoid, and that the deeper of the two adductors migrated downward from origin near the scapula.

The fact that two different components of the brachial plexus pierce the fin base from opposite borders is curious. At present it cannot be incontrovertibly explained. It might suggest distal muscle fibers between the bases of the dermatrichia, but all tissue from this region was found to be of the more fibrous, connective sort. Neither are they sensory nerves, for electrical stimulation of each resulted in adduction of the corresponding half of the fin. One is forced to assume, therefore, that they represent somitic elements that have fused to the distal ends of other muscle elements. The innervation at the extreme distal part of the muscle is, however, somewhat remarkable.

Another detail needing explanation is the fact that these nerves to the fin borders pass between the two layers of fin rays. The nerve situation indicates that the dermal elements of the fin, later becoming dermatrichia, always have been double, or at least partially double, since the time when there was first attachment of somatic slips to poorly differentiated dermal scutes in the situation of the future fins. The inference suggested is either that the dermatrichia of teleosts are derived from double, longitudinally parallel lines of dermal scutes, which does not seem altogether likely, or else that the single line of scutes representing the profin had double bases, projecting dorsally and ventrally to a slight

extent, so that as the scutes lengthened the separation of their bases became first accentuated and then complete.

SUMMARY

Although teleosts are highly specialized in many respects, the nerve, muscle, and skeletal features of such a representative as the cod (*Gadus*) offer much evidence of value to the student of tetrapod morphogenesis. Four brachial nerves innervate adductor (extensor) and abductor (flexor) groups of muscles, each group being composed, typically, of a main dorsal, more superficial, and a more ventral component, each of the four muscles inserting upon the whole width of the fin base. The dermatrichia occur in two layers, between which, from each border, extend branches of the brachial nerves. This indicates that the double character of at least the bases of the fin rays must have been established from the very beginning of fin differentiation.

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STUDIES OF THE MITOTIC FIGURE

II. SQUALUS: THE BEHAVIOR OF CENTRAL BODIES IN BRAIN CELLS OF EMBRYOS

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TWENTY-SEVEN FIGURES

INTRODUCTION

Purpose of the study

In 1929, Pollister described mitosis in cells of the pancreatic acini of the smooth dogfish, *Mustelus canis*. He reported the behavior of the various cell components, but especially that of the central bodies, where his chief purpose was to determine whether or not the conclusion reached by Fry in his study of *Echinarachnius* eggs ('29)—that the central body is a coagulation artifact of the area of focalized rays—is applicable to dogfish cells. He concluded (p. 41) that "the structures described as 'pleuricorpuscular centrosomes' . . . in such cells [*Echinarachnius*] may very well be artifacts caused in the exact way Fry describes, but it appears to be highly improbable that this fact has any significance with relation to problems concerning typical central bodies in epithelial cells."

The present paper reports the results of further studies of centrioles in dogfish cells. The data are first presented and discussed without reference to Pollister's findings, after which the observations and conclusions of the two studies are compared.¹

¹ Appreciation is expressed to Professor Pollister for his loan of slides of his material and for reading the manuscript of the present paper. A grant from the National Research Council facilitated the completion of the investigation.

Materials and methods

In the spiny dogfish, *Squalus acanthias*, which was used in this investigation, dividing cells were found to be so rare in the pancreas as to make the study of mitosis in such material impracticable, although this organ was fixed and examined in eight individuals. However, mitotic figures were found to be abundant in embryos 4 to 7 cm. long, and they were therefore used for the experiments. The cells of the ependymal layer, lining the brain cavities, were selected for study for several reasons: this tissue has a higher percentage of mitotic figures than any other, having from 5 to 50 per cent of the cells in division; there are no cytoplasmic granules which can be confused with centrioles; and the brain constitutes so large a proportion of an embryo that a number of slides containing such tissue can be secured from it.

Embryos were removed from the uterus immediately after pithing and opening the female, and were at once dissected from the yolk sac and surrounding membrane. Some were then promptly dropped into various reagents in order to study the effects of different fixatives upon the mitotic figure; others were immersed for half an hour in modified sea water prior to fixation, in order to study the effects of different environmental factors.

All material was sectioned at a thickness of 5μ and stained with Heidenhain's haematoxylin. Since the depth of stain modifies radically the appearance of the centriole, this factor was kept as constant as possible, in the following way: Three to five slides were made from each variously treated embryo and destained to different degrees; a moderately stained slide, made from a Bouin-fixed embryo, was selected as a standard; slides of the other embryos were compared with it, and in each case only one similarly stained was studied—except in the experiment dealing with the effects of different depths of stain.

Since preliminary experiments showed that various common reagents, including Bouin's, demonstrate similar centrioles, that fluid was selected for use, except in the experiments which deal with the effects of different reagents.

Under each experimental condition—varied as to mitotic phase, fixation, environmental factors, or depth of stain—at least twenty-five mitotic figures were studied carefully; many more were observed briefly. Under any given condition, all division figures were usually found to be alike; exceptions have been noted.

Each illustration (enlarged 2500 times) has been drawn from a single cell selected because it was typical of its class in all respects. The sizes of the smaller centrioles are necessarily only approximate, since a considerable error is involved in measuring such small objects with an ocular micrometer, even under critical lighting conditions.

The terminology offers no difficulties, for in dogfish tissues there is present only a minute, granule-like dark body, which is generally called a centriole.

EXPERIMENTS

*Part I. The behavior of centrioles from prophase to telophase under optimum conditions: normal environment;
Bouin's fixation; medium depth of stain*

Behavior during prophase. When the figure is first discernible, it apparently has the following structure: a single round black centriole, about 0.4μ in diameter, sharply outlined, lies somewhere on the line where the nuclear membrane existed a little earlier; from it the distinct fibers of a half-spindle, which may lie in any axis of the cell, extend into the tangled mass of chromosomes (fig. 2). No earlier stages, to indicate how the half-spindle arises, were found, nor was any stage intermediate between this and the fully formed metaphase figure discovered.² At no time are asters present.

² The failure to find such transitional conditions, in spite of the examination of many prophase cells, is probably explained by the difficulties involved in studying this phase. For example, the absence of asters hinders the detection of the earliest stages in the formation of the mitotic figure. Furthermore, since the division figure lies entirely within the nucleus, its details are difficult to distinguish among the tangled prophase chromosomes. Again, since the cells are about $10 \times 11\mu$ in size, and the material is sectioned at a thickness of 5μ , it

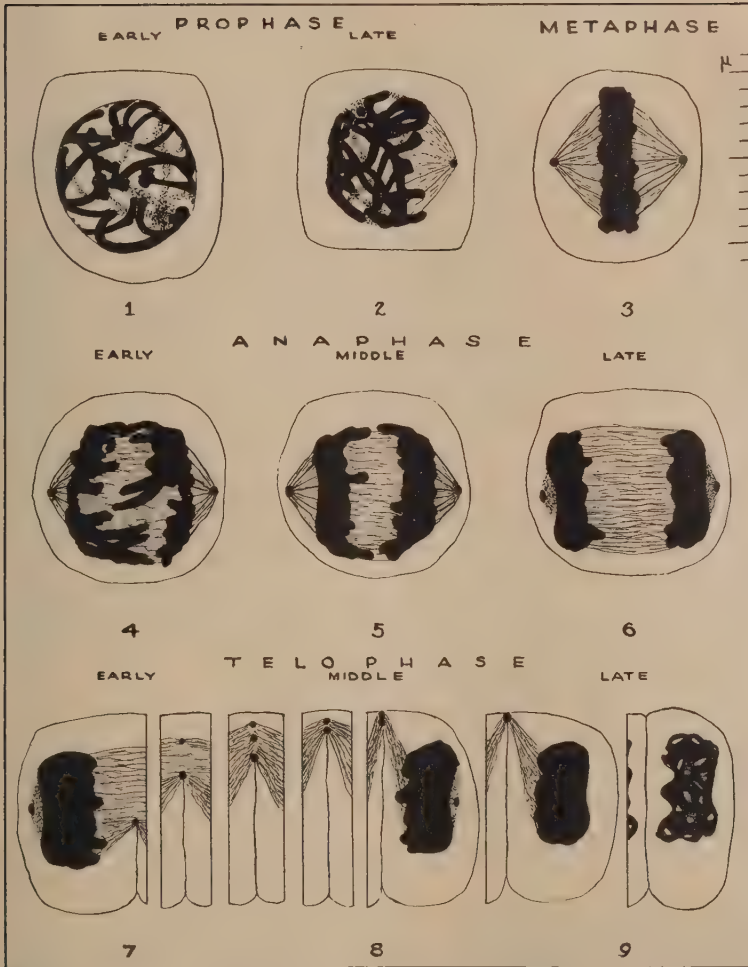
In early prophase cells (fig. 1), in which spindle fibers have not yet arisen, it is impossible to ascertain whether or not a centriole is present, since cross sections of chromosomes and irregularities along the nuclear membrane frequently simulate centrioles. Hence it is not known whether the centriole exists prior to the origin of the half-spindle with which it is later associated, or arises simultaneously with it.

Behavior during metaphase. At this stage (fig. 3) the appearance of the centriole is similar to that at prophase, although the spindle fibers are somewhat more distinct. The spindle end forms an angle of about 90° , and the long axis of the figure may lie in any position in the cell.³

Behavior during anaphase and telophase. When the chromosome groups separate during anaphase and approach the centrioles, the angle of each spindle end widens and the centriole enlongates; the fibers remain distinct (figs. 4 and 5). At telophase the spindle end has an angle of about 140° , and the centriole shows its maximum elongation. By this time the chromosome group has become concave on the side toward the centriole, and the latter lies near the concavity. The fibers of the spindle end gradually fade, until only a homogeneous cone of material is left. When the cone disappears the centriole disappears with it, although the middle region of the spindle still shows distinct fibers (figs. 7, 8, and 9). These are gradually pinched together by the cleaving cell, and midbodies appear at points where they are focalized; these will be discussed later.

frequently happens that a peripheral part of the cell, possibly containing important evidence, lies in a neighboring serial section, where it is difficult to locate among the irregular configurations of such tissues. Hence the observations reported here concerning prophase are to be regarded as tentative. This applies especially to the apparent origin of the mitotic figure as a half-spindle. This stage is found but rarely, and it is possible that the other spindle end is present, but is invisible among the crowded chromosomes, or that it occurs in a neighboring serial section where it was not found despite a search for it.

³One hundred figures, sectioned so as to be seen in side view, were chosen at random for study. In 40 per cent of them the figure lies in a line parallel to the cell surface bordering the lumen of the brain vesicle; in another 40 per cent it lies obliquely; in 20 per cent it is at right angles.



Figs. 1 to 9 The behavior of centrioles and mid-bodies in brain cells of *Squalus* embryos during mitosis. Bouin's fixation; medium depth of stain (Heidenhain's haematoxylin). Drawings are enlarged $\times 2500$. Result: The centriole does not divide; its shape changes with that of the spindle end; it disappears when the spindle end fades, although the middle region still persists. Mid-bodies arise when the mid-fibers are focalized by the cleaving cell; their number and size are related to the manner in which the fibers are grouped; mid-bodies and fibers disappear simultaneously. The structure of both centrioles and mid-bodies is related to the detailed configuration of the focalized fibers with which they are associated; both are probably phenomena of focalization.

Three conclusions may be drawn from these data: 1. *The centriole does not divide during mitosis.* At all mitotic stages every spindle tip has but one centriole; not a single exception was found. If successive generations of centrioles in these cells arise by the division of pre-existing ones, thus exhibiting genetic continuity, such division occurs during the interkinetic period, when no evidence on the point can be obtained.

2. *There is probably some relation between the presence and absence of the spindle end and the presence and absence of the centriole.* This is indicated during telophase, when both apparently disappear simultaneously, although the middle region of the spindle still persists.⁴ There is no possibility of confusing the centriole with cytoplasmic granules, for no such granules are present in brain cells. If the centriole persisted for even a short time after the disappearance of the spindle end, it would be easily located, since it lies near the concavity of the chromosome group (figs. 8 and 9). It is possible that it migrates into the chromosome mass just at this time; or it may become submicroscopic or change its chemical composition so as to become non-demonstrable. Otherwise, the disappearance of the centriole is related to the disappearance of the spindle end.

3. *The shape of the centriole is related to the shape of the spindle end.* During prophase and metaphase, when the spindle end has a relatively sharp angle of about 90° , the centriole is round. During anaphase and telophase, when the spindle end widens to an angle of about 140° , the centriole elongates in the same direction as that in which the spindle end widens.⁵

⁴ At other times in the cell cycle, evidence concerning this relationship is not available: during the interkinetic period, and during early prophase, before the spindle arises, centrioles, if present, are indistinguishable among the nuclear materials; the fact that they are distinct from early prophase to middle telophase, when the spindle ends are also present, gives no evidence concerning whether or not the centrioles can exist in the absence of the spindle ends.

⁵ A single metaphase figure was found in which one spindle end is sharply focused in the usual manner and the centriole is round, while the other end is shaped like a half-circle and its centriole is distinctly elongate.

Part II. The behavior of centrioles at metaphase under modified conditions

This phase of the study was designed to secure evidence on the possible relation between the detailed structure of the individual spindle fibers and that of the centriole, apart from the relation between the shape of the spindle end as a whole and that of the centriole. This possibility was suggested by the discovery that in *Chaetopterus* eggs there is a relation between the morphology of the central body and the coarseness and shape of the rays (Fry, '32). The previous facts suggest that in the brain cells of *Squalus* there is no such relation, since the centriole is equally distinct whether the fibers are distinct, as in prophase, metaphase, and anaphase, or are vague, as in telophase.

Three groups of experiments were carried out to secure further information on this point, each modifying a different phase of the technique in order to bring about changes in the mitotic figure, and thus to determine whether or not changes in the fibers are related to changes in the centriole. The study was confined to metaphase, because, under optimum conditions, that is the time when the fibers are coarsest and spindle ends largest and most readily studied.

Group A. The effects of modifying the fixation (normal environment; medium depth of stain). In the three experiments reported here, the embryos were fixed immediately upon removal from the adult, and slides were stained to a medium depth. The techniques of the experiments are described first, and then the results.

1. Use of different fixatives. The reagents employed in this experiment are listed below; formulae found in the ninth edition of Lee's "Microtometist's Vade Mecum" are indicated by references to page numbers; others are given here.

Alcohol-acetic	Absolute alcohol, 99 pts.; glacial acetic acid, 1 pt.
Bouin	Lee, p. 63.
Burchardt	Lee, p. 42.
Champy (modified)	Lee, p. 353.
Chloroform-acetic	Saturated solution chloroform, 99 pts.; glacial acetic acid, 1 pt.
Flemming (strong)	Lee, p. 37.
Flemming without acetic	Lee, p. 332.
Formaldehyde	5 per cent solution.
Gilson	Lee, p. 49.
Hermann	Lee, p. 39 (using 4 cc. of 2 per cent osmic acid solution).
Picro-acetic (Boveri)	Lee, p. 56.
Platinic chloride acetic	1 per cent solution platinic chloride, 99 pts.; glacial acetic acid, 1 pt.
Corrosive sublimate	Saturated solution corrosive sublimate.
Sublimate-acetic	Saturated solution corrosive sublimate, 97.5 pts.; glacial acetic acid, 2.5 pts.
Zenker	Lee, p. 50.

2. Modification of the hydrogen ion concentration of Bouin's fluid. Embryos were fixed in Bouin's reagent at various hydrogen ion concentrations, the pH determinations being made electrometrically by Mr. Delafield Dubois. The formulae are as follows:

40 cc. Bouin's fluid plus	0 cc. N NaOH = pH 1.7
40 cc. Bouin's fluid plus	5 cc. N NaOH = pH 3.9
40 cc. Bouin's fluid plus	10 cc. N NaOH = pH 4.4
40 cc. Bouin's fluid plus	15 cc. N NaOH = pH 4.8
40 cc. Bouin's fluid plus	20 cc. N NaOH = pH 5.2

3. Dilution of Bouin's fluid with distilled water. Because of the marked effects produced upon the central body structure of Chaetopterus eggs by a similar treatment (Fry, '32), Squalus embryos were fixed in Bouin's reagent diluted with 50, 75, and 90 parts of distilled water.

The results of these three experiments are presented in table 1. Three classes of spindles occur: 1) those with distinct fibers; 2) those in which there is a vague linear organization, but individual fibers cannot be discerned; and, 3) those in which the spindle area shows no linear organization whatever. There are likewise three classes of centrioles: 1) in

TABLE 1

Types of metaphase figures occurring in brain cells of Squalus embryos when fixation and environmental factors are modified

METHOD OF MODIFYING THE METAPHASE FIGURE	STRUCTURE OF THE METAPHASE FIGURE			
	Type I	Type II A	Type II B	Type III
	Fibers distinct; centriole distinct (cf. fig. 14)	Fibers vague; centriole vague (cf. fig. 20)	Fibers vague; centriole absent (cf. fig. 23)	Fibers absent; centriole absent (cf. fig. 26)
Group A. Modified fixation; normal environment; medium depth of stain				
1. Use of different fixatives	Bouin Burchardt Chloroform-acetic Flemming* Gilson Hermann Picro-acetic Plat. chlor. acetic* Sublimate-acetic Zenker	Champy Flemming without acetic*	Alcohol-acetic	Formaldehyde 5 per cent Sublimate
2. Modification of the hydrogen ion concentration of Bouin's fluid	Bouin at pH 1.7, 3.9, 4.4, 4.8			Bouin at pH 5.2
3. Dilution of Bouin's fluid with distilled water	Bouin at 50 per cent strength	Bouin at 25 per cent strength*	Bouin at 10 per cent strength*	
Group B. Modified environment; Bouin's fixation; medium depth of stain				
1. Etherizing the sea water	Ether: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 1.0, 2.0 per cent			
2. Modification of the osmotic pressure by diluting the sea water with distilled water	Sea water at 90, 80, 70, 60, 50 per cent strength			
3. Modification of the temperature of the sea water	10°, 20°, 30°		35°*	39°*

Result: The structure of the centriole is related to that of the spindle fibers: if fibers are distinct, centrioles are distinct; if fibers are vague, centrioles are vague or absent; if fibers are absent, centrioles are absent. (An asterisk signifies that, although the majority of the metaphase figures are of the type indicated, some of them belong to one or the other or both of the neighboring types.)

some cells they have a distinct contour and are darkly stained; 2) in some their contours are indistinct and they stain lightly; and, 3) in others they are absent.

When metaphase figures are modified by various reagents there is a definite relation between the distinctness of the spindle fibers and of the centriole: when fibers are distinct, centrioles are distinct (type I, table 1); when fibers are vague, centrioles are either vague (type II A) or absent (type II B); and when fibers are absent, centrioles are absent (type III). These various types are illustrated in chart 1, p. 170. In most cases, all metaphase figures in the brain cells of any one embryo are of the same type; in a few, designated in the table by an asterisk, a limited number of the cells belong to either or both of the neighboring types, although most of them belong to the class indicated.⁶

Group B. The effects of modifying the environment (Bouin's fixation; medium depth of stain). In these three experiments each embryo was immersed for half an hour in sea water, variously modified as described below, after which it was fixed in Bouin's reagent. The slides were stained to a medium depth. Results of the experiments are summarized after the technique used in each is described.

1. Etherizing the sea water. The following percentages of ether were added to sea water at 22°C.: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 1.0, and 2.0. An embryo was immersed in each solution. When they were picked up for transfer to the reagent, they all wriggled, except the one in the 2.0 per cent solution, indicating that, with one exception, they were not in a moribund condition as a result of the treatment.

⁶ This relationship between the structure of the spindle fibers and that of the centriole holds true in about 98 per cent of the cells. The few exceptions are found only in cells with vague or delicate fibers, which are transitional between those having typically coarse fibers and those with none at all. It is probable that the inability to relate the structure of the centriole to that of the fibers in these transitional cases is due not only to the difficulty in making accurate observations in mitotic figures where a half-spindle is only 4μ long, but also to the radical modification of the appearance of the centriole caused by slight differences in depth of stain, which will be demonstrated later.

2. Modification of the osmotic pressure by diluting the sea water. Sea water at 22°C. was diluted with 10, 20, 30, 40, and 50 parts of distilled water. Again, only the embryo immersed in the last of these failed to wriggle when picked up for fixation.

3. Modification of the temperature of the sea water. Embryos were placed in sea water at 10°, 20°, 30°, 35°, and 39°C. Corrected thermometers were used and the temperature did not vary more than 0.5° in each case. Only the embryo exposed to a temperature of 39° failed to move at the time of fixation.

After modifying the environmental factors, the same types of metaphase figures occur as those demonstrated after using various fixatives (table 1). The same relationship between the distinctness of the spindle fibers and that of the centriole is again apparent.⁷

Group C. The effects of modifying the depth of stain (normal environment; modified fixation). The purpose of this group of experiments is to determine the effects of different depths of stain on the relation between the physical structure of the centriole at metaphase and that of the spindle fibers. The conditions existing when the various metaphase types are stained lightly, moderately (to a medium degree), and darkly, are presented in chart 1 (figs. 10 to 27).

⁷If a given exposure to an environmental modification does not permanently harm the cells—in other words, if they would return to their normal structure when returned to a normal environment—then any changes produced in the mitotic figure are due to the specific environmental factor concerned. If, however, the changes produced in the cells lead sooner or later to death, the resulting modifications of the mitotic figure may be those associated with cytolysis, regardless of what environmental factor brought it about. In many studies it would be necessary to determine which of these two factors is involved, but in the present case this is immaterial, since the purpose is to modify the division figure to see if changes in the spindle fibers are related to changes in the centriole, and the method of producing them is unimportant. That lethal conditions were not usually involved, however, is indicated by the fact that after the half-hour treatment each embryo wriggled when picked up for transfer to the fixative, behaving like those just removed from the uterus; the few instances when embryos were rendered immobile are noted in the experiments. The question might also be raised, whether changes due to asphyxiation are involved; this can be ignored, since the mitotic figures of a control embryo immersed in normal sea water for half an hour prior to fixation are similar to those in embryos fixed at once.

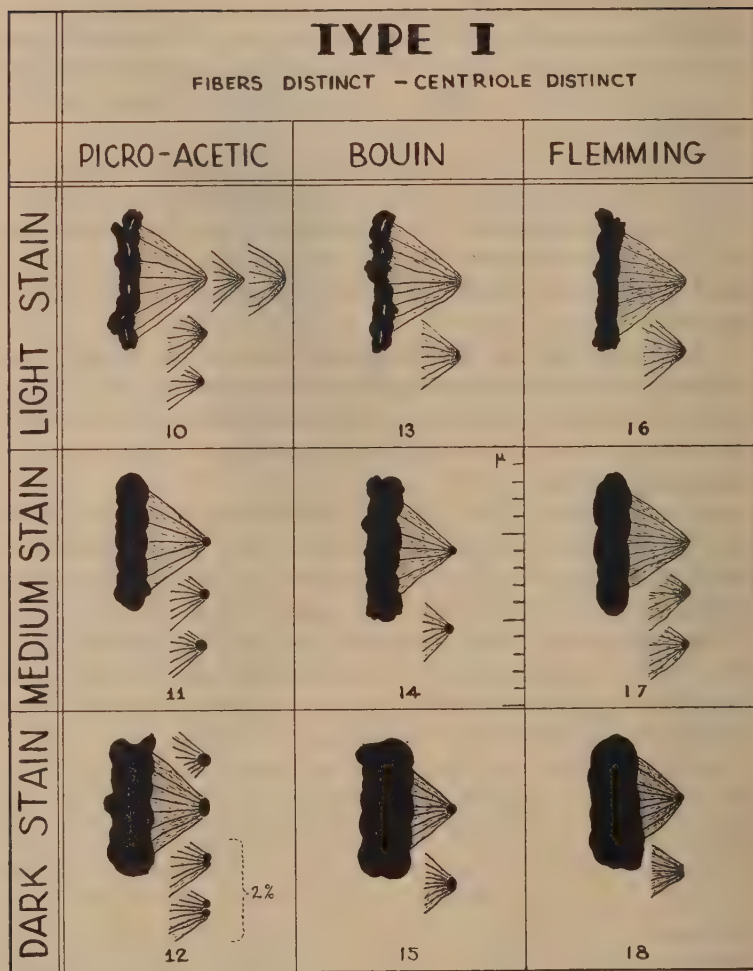


Chart 1 (figs. 10 to 27) The structure of centrioles in various types of metaphase figures in brain cells of *Squalus* embryos, as modified by different depths of stain with Heidenhain's haematoxylin. The various metaphase types are produced by using different fixatives. Drawings are enlarged $\times 2500$. Result: The darker the stain, the more chance is there that any metaphase figure, regardless of type, has centrioles, and the larger they are. In darkly stained cells they are frequently elongate, and rarely bipartite (2.0 per cent). Typical centrioles occur only if

TYPE II A FIBERS VAGUE CENTRIOLE VAGUE	TYPE II B FIBERS VAGUE CENTRIOLE ABSENT	TYPE III FIBERS ABSENT CENTRIOLE ABSENT
CHAMPY	ALCOHOL-ACETIC	FORMALDEHYDE
 19	 22	 25
 20	 23	 26
 21	 24	 27

fibers are distinct and a certain minimum depth of stain is used. They disappear as cells are destained, the spindle fibers continuing to make contact with them at every stage in the process, until nothing is left but the fibers. Thus the structure of metaphase centrioles is related to that of the fibers, coupled with the depth of stain; they are probably accumulations of dye at points where fibers converge, i.e., focal staining artifacts.

Under each experimental condition the spindle end in different cells of the same embryo may be sharply focused, slightly blunt, or very blunt, as shown in figure 10; the other illustrations usually show the slightly blunt shape only, since it is most common.

Three embryos represent type I, which, when moderately stained, has both distinct fibers and centrioles: the first, fixed in Boveri's picro-acetic reagent, shows the largest centrioles occurring in the previous experiments, about 0.6μ in diameter; the second, fixed in Bouin's fluid, shows centrioles of average size, about 0.5μ in diameter; the third, fixed in Flemming's fluid, illustrates an intermediate type, i.e., the fibers are distinct, although delicate, and the centriole is distinct, vague, or absent. Type II A is represented by an embryo fixed in Champy's fluid, which shows the vague fibers and centrioles typical of that class when moderately stained. Type II B, with vague fibers and no centriole when moderately stained, is illustrated by an embryo fixed in the alcohol-acetic mixture previously used (p. 166). Type III, which under optimum conditions shows neither fibers nor centrioles, is illustrated by an embryo fixed in a 5 per cent solution of formaldehyde. The embryos selected for illustration exhibit the major structural variations at metaphase brought about by modifying the depth of stain in all of the variously treated embryos previously reported.

Five to ten slides were made from each representative embryo and stained to different degrees with Heidenhain's haematoxylin, varying from an extremely dark to an extremely light condition. The appearance of the metaphase chromosome plate constitutes a measure of the depth of stain: in overstained material it is thick (about 3μ) and the individual chromosomes are completely indistinguishable; in lightly stained cells the plate is thinner (about 2μ or less) and the individual chromosomes are discernible to various degrees (chart 1).

The results are as follows: 1. Regardless of type, the darker the stain the more likely the cell is to have centrioles.

When the stain is dark, all cells have them, with the exception of a few belonging to type III; when cells are moderately stained, most of those with distinct fibers (type I) and some with vague ones (type II) have centrioles, but the others do not; when the stain is light, only a few of the cells with distinct fibers (type I) have them.

2. Regardless of type, the darker the stain, the larger the centriole is.

3. When slides are destained to various degrees, centrioles hold the stain in proportion to the distinctness of the fibers: figures without fibers (type III) are the first to lose the stain at the spindle tip; those with vague fibers (type II) give it up next, and finally those with distinct ones (type I).

4. Regardless of the depth of stain, the extent to which the centriole is similar in size and shape in the cells of a given embryo is proportional to the distinctness of the fibers. *a*) In cells stained darkly, where centrioles are usually present in all types, those associated with distinct fibers (figs. 12, 15, and 18), and with vague ones (figs. 21 and 24) are similar in size and shape in the metaphase cells of any one embryo. On the other hand, in cells without fibers (fig. 27) there is great variability in centriole structure: occasionally they are absent; their size varies from 1.2μ down to the limits of visibility; they may be of any shape. *b*) In material stained moderately, as previously noted, typical orderly centrioles usually occur only if fibers are distinct (figs. 11, 14, and 17); if they are vague, the centrioles are vague (fig. 20) or absent (fig. 23); and they are always absent if fibers are absent (fig. 26). *c*) When cells are lightly stained, orderly centrioles occur within a still narrower range, being found only in some of the embryos having distinct fibers (fig. 10).⁸

5. In heavily overstained material, some of the cells have oval centrioles, the long axes of which are at right angles to that of the spindle (figs. 12, 15, and 18). Under conditions of overstaining there may occur rarely (in 5 cells out of about

⁸ During anaphase and telophase the conditions are similar to those occurring at metaphase: that is, the deeper the stain, the larger the centriole, and centrioles are absent when slides are lightly stained.

200 overstained ones studied) a bipartite centriole, like two spheres, partially fused or quite separate (fig. 12). Such elongate or bipartite centrioles cannot be interpreted as stages in division, demonstrable only when the material is heavily stained, since, if such were the case, they would be present as double bodies during anaphase and telophase—a situation that is never found, no matter what the depth of the stain. These elongate and bipartite metaphase centrioles are apparently the result of overstaining the spindle end.

This study of the effects of various depths of stain upon the appearance of the centriole in the different metaphase types indicates that (under any given set of experimental conditions) we are not dealing with a cell component of definite size. For example, when an embryo fixed with a micro-acetic reagent is deeply stained, the centriole is 0.8μ in diameter and sharply outlined, and the spindle fibers make contact with it (fig. 12). It may be that a heavy stain is especially favorable for demonstrating this centriole, but if it is a delimited body 0.8μ in diameter, at some stage in the process of destaining it should be visible as a lightly stained body, still 0.8μ in diameter, even though it may eventually disappear as progressive destaining extracts the dye from it. What actually happens is quite different: *During progressive destaining the body is equally black at every stage, but it becomes smaller and smaller, and the fibers continue to make contact with it until it disappears and nothing is left but converging fibers* (figs. 10, 11, and 12). This is also true of the other variously fixed embryos. In other words, the structure at the spindle end does not behave like an individualized body of specific size, such as a chromosome, but rather like an accumulation of dye at the spindle end, which is progressively reduced in size as the slide is destained, until it disappears. Furthermore, if the fixed fibers have a distinct organization, this accumulation of dye presents an orderly appearance as to shape and contour, but its structure is variable if the linear organization of the spindle is vague or lacking.⁹

⁹ It is possible that, in addition to the dye at the spindle end, a sub-microscopic body may be present. If so, it is beyond demonstration.

Comparison of the data of parts I and II

A relation between the structure of the spindle fibers and that of the centriole—the distinctness of one being related to that of the other—is clearly apparent at metaphase when the experimental technique is modified as to fixation, environment, or depth of stain (part II). But this relation does not hold true when the behavior of the centriole is studied under optimum conditions from prophase to telophase (part I), where its structure appears to be unrelated to that of the spindle fibers; here it is distinct whether the fibers are distinct, as they are during prophase, metaphase, and anaphase, or are vague, as they are during telophase.

A similar discrepancy occurs also when the different types of metaphase figures are stained to various depths (chart 1): When those with distinct fibers are moderately stained, the centriole is about 0.5μ in diameter (figs. 11, 14, and 17); but when they are darkly stained, it is about 0.7μ in diameter (figs. 12, 15, and 18). On the other hand, when metaphase figures showing no spindle fibers are moderately stained, the centriole is absent (fig. 26); but when they are darkly stained it is frequently 1.0μ or more in diameter (fig. 27). In other words, if metaphase figures have distinct fibers, then increasing the depth of stain from medium to dark causes only a slight increase in the size of the centriole which was already present; but if fibers are absent, a corresponding increase in the depth of stain frequently results in the demonstration of a very large centriole which was not shown previously.

Similar anomalies have arisen in connection with studies of other material, as yet unpublished. For example, in *Echinarachnius* eggs, when Bouin's fluid is used, the large asters of the first cleavage figures have large central bodies and the smaller asters of the later cleavage figures have minute ones, as might be expected; but when *Cerebratulus* eggs are fixed with the same reagent, the situation is reversed: the large first cleavage asters have minute central bodies and the smaller asters of late cleavage have large ones. Thus the relation between the size of the central body and the

size of the aster during segmentation may differ from species to species. Again, when *Cumingia* eggs are fixed with various reagents, the central body phenomena in the relatively large mitotic figure of the first maturation division are very different from those of the immediately succeeding smaller figure of the second division.

These facts cannot be explained at the present stage of our knowledge. It is possible, however, that in many cases (excluding blepharoplast-centrioles) the changes in central body structure—which may differ from species to species at the same stage in their life history, or from one cell cycle to the next within the same species, or from one mitotic phase to the next within the same mitotic cycle—may eventually be explained in terms of changes in the detailed configurations of converging spindle fibers and astral rays, and these in turn are determined by such phenomena as cell size, width of the chromosome plate, and the physico-chemical structure of the cytoplasm. These relationships are further modified by environmental factors and the action of the coagulating agents. It will require many studies of diverse cell types before there is an understanding of the intricacies of the relationship which exists in some cells between the morphology of the central body and that of fibers and rays.

DISCUSSION

In a recent study of *Chaetopterus* eggs, various classes of centrioles and centriole-like bodies were discussed, each distinguished by various characteristics (Fry, '32, p. 181). In determining to which of these categories the centrioles of the present study belong, certain ones can be dismissed at once. Since central bodies of *Squalus* brain cells have no structures concerned with motility, they are not blepharoplast-centrioles; nor are they random granules—the presence of a single body at each spindle tip eliminates that possibility; nor are they diplosomes, which occur in certain epithelial cells, since the bodies of the present material do not occur as bipartite

structures lying in the cytoplasm during interkinesis.¹⁰ It seems equally probable that they are not typical centrioles, for they do not exhibit a relatively stable size and shape regardless of whether the rest of the achromatic figure is present or absent, and they are affected by changes in the exact configuration of the fibers, and they are extensively modified by differences in the depth of stain.

Two other classes of centriole-like bodies remain to be considered—focal bodies and focal staining artifacts, both of which are phenomena of the focalization of fibers or rays, existing only where fibers or rays converge. They differ in the following respects: a focal body exists in the coagulated cell as a non-radial structure of specific size (regardless of whether or not it existed in life, or is the result of coagulation), and it maintains its characteristic size in spite of wide variations in the depth of stain somewhat as does a chromosome. A focal staining artifact, on the other hand, is nothing but an accumulation of dye at the area of focalization, and its size is therefore modified by relatively minor changes in the depth of stain. The centriole of *Squalus* brain cells appears to be a phenomenon associated with the focalization of fibers, and it is probably a focal staining artifact rather than a focal body, since progressive destaining makes it gradually smaller, the spindle fibers continuing to make contact with it at every stage in the process until it disappears, leaving only the focalized fibers.

On a priori grounds it may seem improbable that the point of focus of converging rays or fibers could, under certain conditions of fixation and staining, appear to be a minute, sharply demarked body simulating a centriole. That such may be the case, however, is strongly suggested by the behavior of mid-bodies (*Zwischenkörper*) in the present study. As already noted, the fibers in the middle region of the spindle remain distinct, while those at the ends are fading, and these distinct mid-fibers are gradually pinched together by the cleaving cell. Before this process begins, no mid-bodies

¹⁰ The diplosomes and other types of central bodies described by Rio-Hortega ('16) in nerve cells of mammals do not occur in ependymal cells of *Squalus* embryos.

are visible (figs. 5 and 6), but after cleavage has proceeded a short distance they arise where groups of fibers are brought to a focus; they vary in number, size, and staining capacity, apparently according to the manner in which the fibers happen to be focalized (figs. 7 and 8). The constriction arises at one side of the cell and passes through to the opposite surface, thus pushing the fibers of the mid-region to one side. During the earlier phases of this process, the fibers on the side of the advancing furrow are focalized to a greater extent than those on the other side—and during this period the mid-bodies on the side of the approaching furrow are larger than are those on the opposite side (figs. 7 and 8). As constriction progresses, the mid-bodies become larger and more definite, still showing variations in their detailed structure. When the cell is completely cleaved, and the fibers are thereby focalized to a single point, there is but one mid-body, which shows but slight variations (fig. 9). It persists as long as the spindle fibers persist, and it disappears when they disappear (fig. 9).

If these mid-bodies are to be interpreted as cell components which existed in life as individualized structures, we must assume that for some reason they arise only when the spindle fibers begin to be constricted during cleavage, that they differ in number and size according to the way in which the fibers are focalized, and that they disappear simultaneously with the fibers. Is it not more plausible to conclude that these bodies are artifacts resulting from the coagulation of focalized fibers?

The mid-bodies which are present when the fibers are focalized to a single point look like centrioles. Like them, they are distinct, darkly stained, and relatively similar in structure from cell to cell if the fibers are distinct; when fibers are vague, they are vague or absent. Furthermore, their size is proportional to the depth of stain, and they disappear completely as slides are destained.¹¹

¹¹ Whether or not mid-bodies generally will eventually be explained in terms of focalization phenomena remains to be seen. A number of them have been reexamined, and a report is now in preparation. It is probable that they fall into two major categories: one, an example of which occurs in the present study,

It is therefore concluded that in brain cells of Squalus embryos both the so-called centrioles and the mid-bodies are probably focal staining artifacts, i.e., accumulations of dye at points where fibers converge, whether at the spindle ends or at the middle region, and that they have no existence as individualized cell components in the living cell. This assumption explains why, during telophase, the centriole disappears with the spindle end: a point of focalization creates a place where a focal staining artifact can occur, and its disappearance makes the existence of such an artifact impossible. Second, it explains why the centriole elongates when the spindle end widens: the shape of the cone of converging fibers determines the shape of the mass of dye held at the focal point. Third, it explains why, after experimental modifications, the structure of the centriole is related, at metaphase, to the physical structure of the fibers, as determined by the type of fixation and the depth of stain: the morphology of the converging fibers determines the manner in which the dye is held. Fourth, it explains why the centriole is never oval if a medium depth of stain is used, but is frequently so if cells are deeply stained: most of the spindle tips are somewhat oval; if a minimum amount of dye is used, only the central group of converging fibers, which usually comes to a point regardless of the shape of the spindle end as a whole (fig. 10), retain the stain, and the result is a round body; but if the dye is very abundant, all of the fibers are involved, and the result is an oval accumulation of dye, which is occasionally bipartite, depending upon the exact configuration of the fibers. Fifth, if the body is an accumulation of dye it would naturally not become double unless the spindle end widened extensively during mitosis or its tip became bifocal, which does not occur

behaves like the class of centriole-like bodies called focal staining artifacts; the other behaves like focal bodies, being present as an actual body in the coagulation product regardless of the depth of stain. A modification occurs in insect eggs where there is no cleavage during the earlier stages and hence no focalization of fibers from that cause; bodies nevertheless appear, their number and structure being related to the detailed configuration of the fibers; they are probably condensations of the material of the fibers arising as the spindle disintegrates.

in these cells. And, finally, the behavior of mid-bodies is explained by this assumption: they, too, are accumulations of dye appearing only as the fibers are focalized by the cleaving cell, and undergoing changes in number and size as the configurations of focalization are modified by cleavage.

In previous studies by the senior author, the concept that structures simulating central bodies may be produced by the technique at points of focalization has been applied to areas where astral rays alone converge, as in cytasters or sperm asters of *Echinarachnius* (Fry, '28 and '29), and to areas where both astral rays and spindle fibers converge, as in cleaving eggs of *Echinarachnius* and *Chaetopterus* (Fry, '29 and '32). The present study of *Squalus* cells indicates that similar bodies occur at areas where spindle fibers alone converge, either at the end of the spindle, where they involve the presence of centrioles, or at its mid-region, where they involve the presence of mid-bodies. If this suggestion has general application, then in anastral mitotic figures, such structures of focalization can be present at the spindle ends only if the spindle tip is sharply focalized; if it is blunt, they cannot exist. A study of the illustrations of cytological papers shows that this is generally the case. When the spindles of anastral figures are not sharply focalized, as is the case in most plant cells and in some oocytes and spermatocytes of animals, central bodies are absent (aside from blepharoplast-centrioles). Whether or not the supposed centrioles of anastral mitotic figures which have sharply pointed spindle tips are in many cases artifacts resulting from the focalization of fibers can be determined only by further studies of such figures in various cell types.

This possibility should also be examined in the anastral figures of cancer cells; should it be found that the supposed centrioles in them are also phenomena of focalization, this would eliminate from further consideration any role which they might play in bringing about the neoplastic condition—a possibility which has been suggested by Boveri ('29).

Turning, now, from a consideration of the behavior of centrioles in brain cells of *Squalus* to their behavior in pancreatic cells of *Mustelus*, as described by Pollister ('29), certain similarities and differences between the two studies should be kept in mind: 1. The mitotic figure of *Squalus* cells is without asters; in *Mustelus* cells it has distinct asters during prophase, which fade during metaphase and early anaphase, and are absent thereafter. In both cells the appearance of the chromosome groups, the spindles, and the centrioles is very similar; the distance between spindle tips is also the same (about 8μ), although the *Mustelus* pancreatic cells are larger than the brain cells of *Squalus*. In both, the anastral telophase spindles go through the same changes: the spindle ends fade gradually and finally disappear, while the mid-region persists and shows distinct fibers.

2. Pollister's discussion is organized about a thesis which may be restated somewhat as follows: "In Fry's study of pleuricorpuscular central bodies in *Echinarachnius* eggs he concludes that the central body is only the coagulated area of focalized astral rays. This hypothesis can be tested in pancreatic cells of *Mustelus*, where typical minute centrioles occur, since at various phases in the mitotic process asters may be distinct, vague, or absent." Thus, when Pollister found that the *Mustelus* centriole is distinct, whether the aster is present or absent, he concluded that Fry's suggestion does not apply to typical centrioles in dogfish epithelial cells. At that time, however, neither Pollister nor Fry realized that phenomena of focalization which are associated with converging rays and spindle fibers may also apply to converging spindle fibers without rays.

3. Pollister used four fixatives, but his study of centrioles in pancreas cells is based only on Flemming-fixed tissue (pl. 1, except figs. 8, 9, 14, and 17). His preparations were very deeply stained with Heidenhain's haematoxylin, like those called 'dark' in chart 1 of the present study; he speaks of his slides as "preparations stained as heavily as they must be to demonstrate central bodies" (p. 33); the chromosome

groups are massive and all traces of the individual chromosomes are lost.

His major results which concern those of the present investigation are as follows: 1) In *Mustelus* cells, metaphase centrioles are frequently oval, sometimes bipartite, but, as in *Squalus* cells, none of the anaphase and telophase figures show double ones—a condition which would be expected if the oval shape at metaphase is a stage in the division of the centriole. If the mitotic figures of *Mustelus* react to the dye like those of *Squalus*, elongate centrioles in both species are the result of heavy staining and do not indicate division. 2) During telophase, when the mitotic figures of both species are anastral, centrioles persist in both as long as do the spindle ends, whether fibers are distinct or vague; when the spindle ends fade, the centrioles disappear, even though the mid-region of the spindle is still distinct. 3) In *Mustelus* cells there are indications that the size of the centriole is related to the condition of the rest of the achromatic figure, as it is in *Squalus*. During early prophase, when asters are small, the centrioles are also relatively small; as the asters increase in size, during late prophase, the centrioles do likewise; and when the asters fade during metaphase, the centrioles are somewhat reduced in size, persisting thereafter until the spindle ends fade.

Thus the behavior of the centriole in cells of *Mustelus* is similar in various respects to that in cells of *Squalus*, and both can be explained by the supposition that we are dealing with an accumulation of dye at points where spindle fibers or astral rays converge, and not with an individualized body. Pollister's conclusion that "*in these pancreas cells there is practically conclusive evidence that the central body has an identity distinct from that of either the asters or the spindle*" (p.41) is based upon the appearance of the centriole after using only one fixative, and after staining the preparations very deeply. Whether or not it is a valid conclusion can be determined by studying such cells after using different reagents and thus ascertaining whether or not centriole struc-

ture is modified by changes brought about in the fibers. The use of various depths of stain would also show whether there is a relation between the detailed morphology of the centriole and the amount of dye employed. Such procedure would provide the data necessary to prove whether the centrioles of these two species of dogfish belong to the same or to different categories.

RÉSUMÉ

Centrioles were studied in cells of the ependymal layer of brain tissue in *Squalus* embryos. The technique was modified as to fixation, depth of stain (Heidenhain's haematoxylin), and the environment of the embryos prior to fixation.

The mitotic figure is anastral. The behavior of the centriole is as follows: 1) It does not divide during mitosis; if stained very deeply, it is frequently elongate, and, rarely, bipartite, but these conditions are the results of overstaining and not stages in division. 2) Because of problems of visibility, the only evidence concerning any relation between the presence of the spindle end and that of the centriole is available during telophase, when spindle end and centriole apparently disappear simultaneously, although the mid-region of the spindle still persists. 3) The shape of the centriole is related to that of the spindle tip as a whole. 4) The distinctness of the centriole, at metaphase, is related to the distinctness of the spindle fibers: if fibers are distinct, centrioles are distinct; if fibers are vague, centrioles are vague or absent; if fibers are absent, centrioles are absent. This relationship is modified during telophase. 5) Progressive destaining gradually decreases the size of the centriole, which remains equally black until it is at the limits of visibility; at every stage in the process the spindle fibers continue to make contact with it, and finally nothing is left but the converging fibers.

It is therefore concluded that the centriole is probably nothing but an accumulation of dye, i.e., a focal staining artifact, at the spindle tip where fibers converge; its size, shape, and contour are determined by the detailed configuration of the converging fibers, coupled with the depth of stain. The cen-

triole probably has no existence as an individualized cell component in the brain cells of *Squalus*.

The mid-bodies behave like centrioles. They arise only at points where the fibers of the mid-region of the spindle are focalized by the cleaving cell; their size is modified by the number of fibers converging to a given point. Like centrioles, they are distinct, darkly stained, and have a sharp contour, only if the fibers are distinct; if the fibers are vague, they are vague or absent. Again, like centrioles, their size is proportional to the depth of stain, and they, too, disappear completely if slides are destained. It is therefore concluded that the mid-bodies, like the centrioles, are also focal staining artifacts and have no existence as cell components.

The present interpretation concerning the nature of the centriole in the brain cells of *Squalus* can also be applied to Pollister's data ('29) concerning centrioles in pancreas cells of *Mustelus*.

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A CRITICAL STUDY OF HISTOCHEMICAL METHODS FOR THE DETERMINATION OF IODIDES IN TISSUES¹

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To be able to demonstrate iodides histologically within tissues and tissue cells in the exact location that they occupy during life would be of considerable significance and a great aid in illuminating and clarifying the many obscure aspects of the biologic dynamics of iodides. A technique for so demonstrating iodides was reported by one of us in 1923(10). The procedure, however, was not entirely satisfactory, and the present report is based upon further investigations into the problem.

The technique previously suggested consisted in perfusion with lead nitrate through the aorta to bring about the precipitation of the rather insoluble lead iodide. Fixation was completed by immersion of the blocks of tissue for 24 hours in formalin containing lead nitrate. After sectioning, the tissues were treated with palladous chloride to form brown-black palladous iodide which is far more insoluble than lead iodide. By this method precipitated iodide was demonstrated in the renal convoluted tubules, the interstices of the connective tissue, the walls of Henle's loop, in the nuclei of the hepatic parenchymatous cells, and in the free border of the columnar epithelium of the trachea and bronchi.

Leschke(7) attempted to demonstrate iodides histologically with silver nitrate. He washed the kidneys supposedly free

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of chlorides by the liberal administration of water to a starved rabbit, injected a solution of sodium iodide, and killed the animal 15 minutes later. Small blocks of tissue were fixed in dilute acidulated silver nitrate solution in the dark. After washing the blocks in water they were exposed to light and placed in a solution of a photographic developer. The resulting deposit of silver was claimed to represent iodide.

Hintzelmann(3) has recently suggested immersion of blocks of tissue in dilute formaldehyde solution containing 1 per cent of thallous acetate. After cutting frozen sections, yellow crystals of thallous iodide were seen. The fact that such large aggregates as crystals were built up strongly indicates that diffusion of iodide to the thallous ion must have occurred. The sensitivity of the reaction (1 part in 16,000) does not suffice for histologic work. The studies of Justus(4, 5, 6) upon the histologic distribution of organically bound iodine are not applicable to ionized iodide.

For truly satisfactory histochemical reactions the following criteria must all be fulfilled:

1. The reaction must be sufficiently sensitive to detect extremely minute quantities of the substance tested for; the precipitate must therefore be extremely insoluble.

2. The precipitating reagent must penetrate very rapidly through the tissues, to prevent any appreciable post-mortem diffusion of the substance tested for.

3. The precipitate formed must be visible in thin histologic sections and the color of the precipitate must be specific for the substance under investigation.

Immersion of blocks of tissue for fixation and precipitation of iodides involves very slow penetration of the reagent, which permits of marked post-mortem diffusion of the tissue solutes. Thus, to fulfill the second criterion above, perfusion of the reagent is necessary. In our experiments the precipitating reagent was perfused under a hydrostatic pressure of 4 to 5 feet through the thoracic aorta. The animals were killed by bleeding and the thoracic aorta was cannulated. To avoid interference with the free flow of the reagent through the cir-

culatory system, the arterial tree was first washed out with 20 cc. of isotonic saline solution(1). The aorta was clamped below the renal vessels. The right auricle was opened. It is possible to complete perfusion in from 5 to 7 minutes from the moment of cutting the throat of the rabbit.

EXPERIMENTAL RESULTS

Adult rabbits were used in almost all the experiments; in a few instances adult guinea pigs were employed. Potassium iodide was administered intravenously in doses approximating 0.25 gm. per kilo body weight.

Three general methods were investigated: 1) the precipitation of iodides as such in the tissues; 2) the precipitation of colored insoluble iodides in the tissues, and, 3) the precipitation of colorless or faintly colored insoluble iodides which could later be converted into deeply colored precipitates after sectioning.

1. Attempts to precipitate iodides as such in the tissues

The rapid removal of water and its rapid replacement by a substance in which iodides are insoluble should permit of the precipitation of iodides as such. This might be accomplished by rapid freezing of tissues, thus preventing diffusion of iodides, with subsequent desiccation at low temperatures (2), or by the replacement of water with a solvent in which iodides are insoluble (i.e., ethyl acetate). Typical protocols illustrate the methods of approach investigated.

a. One hour after the injection of the usual intravenous dose of potassium iodide, a rabbit was killed by bleeding. A block of kidney was quickly frozen with carbon dioxide and frozen sections cut with a dry knife. The sections were dropped directly into a 1 per cent solution of palladous chloride (palladous iodide is brown-black and extremely insoluble). No localized browning could be demonstrated; apparently the iodide had diffused out during the manipulations.

b. This method of freezing not being rapid enough, blocks of kidney, stomach, duodenum, and pancreas of another rab-

bit injected with potassium iodide were dropped directly into acetone cooled to $-40^{\circ}\text{C}.$ by liquid air and other blocks into absolute alcohol cooled to $-40^{\circ}\text{C}.$ in the same way. The specimens were then kept at about $0^{\circ}\text{C}.$ until dehydration was complete and were then embedded in paraffin. Sections mounted dry on slides revealed no localized precipitate after being placed in a 2 per cent solution of palladous chloride directly from xylol and absolute alcohol.

c. Tissues were also treated by the Altmann technique(2). In brief, the Altmann technique consists in rapid freezing of tissues in liquid air, followed by complete desiccation in a vacuum at a low temperature ($-20^{\circ}\text{C}.$), so that the water of the tissue never melts, but is removed as vapor directly from the ice in the tissue. Thus only a minimal diffusion of the salt is possible. However, when the completely desiccated tissue was embedded directly in paraffin, sections revealed no localized precipitate of iodide after treatment of the sections with palladous chloride. Apparently the diffusability of the iodide is so great that it diffuses out faster than the reagent can enter, even from thin histologic sections. This impression is confirmed by observing flakes of brown palladous iodide form on the surface of the section and float about when the reaction is carried out on the slide under the microscope.

d. Potassium iodide is soluble in ethyl acetate only to the extent of 0.0013 gm. in 100 gm. at $25^{\circ}\text{C}.$ (9, p. 541). Ethyl acetate is also somewhat miscible with water; it can dissolve 2.35 per cent of water by weight at $0^{\circ}\text{C}.$ and 5.29 per cent at $60^{\circ}\text{C}.$ (9, p. 285). Ethyl acetate hardens tissues effectively and therefore may be used for histologic fixation. Perfusion of 600 cc. of ethyl acetate through the aorta of an animal, previously injected with potassium iodide, was carried out. Small blocks of kidney, stomach, duodenum, and pancreas were placed in ethyl acetate at $-40^{\circ}\text{C}.$, at room temperature, at $37^{\circ}\text{C}.$, and at $62^{\circ}\text{C}.$ Sections from these blocks, after embedding directly in paraffin from the ethyl acetate, revealed no precipitate with palladous chloride.

Under these conditions complete dehydration must be slow (4 to 24 hours), and diffusion of iodide takes place. To reduce the time and diminish the solubility of water in the ethyl acetate, another experiment was performed in which frozen sections of the tissues were cut with a dry knife immediately after the perfusion with fresh distilled ethyl acetate. These sections also failed to reveal iodides histologically.

2. Attempts to precipitate iodides in tissues by perfusion with reagents yielding colored insoluble precipitates

The reagent to precipitate iodides must penetrate the tissues very rapidly and form an exceedingly insoluble precipitate with iodide ion. However, the heavy metal salts which precipitate iodides, also produce a precipitation of proteins. If the rapidity of protein precipitation is greater than that of the iodide, a wall of coagulated protein blocks further penetration of the reagent which permits of post-mortem diffusion of the iodide. Because of this difficulty with penetration with all the reagents studied, it was not possible to develop a truly effective technique for the histochemical detection of iodides. The difficulties encountered in the investigation are in a general way applicable to the problem of precipitating histologically in situ any negative ions, such as iodides, chlorides, bromides, and the like.

Silver iodide is extremely insoluble; 0.000035 gm. of silver iodide being soluble in 100 gm. of solution at 21°C. Silver salts, however, precipitate tissue proteins very rapidly and penetrate with difficulty. Furthermore, perfusion with silver nitrate results in the precipitation of many other salts (chloride, phosphate, albuminate, etc.) and the resulting deposit of black silver is not specific for iodides. It is true that silver iodide is more insoluble than silver chloride and that the latter can be largely removed by immersion of the sections in a saturated solution of sodium chloride, but, at best, the reaction is not wholly specific.

Perfusion of silver nitrate through an animal previously injected with potassium iodide yielded sections with a pre-

precipitate in and around the vascular walls and indiscriminately scattered through the interstitial tissue. Penetration of the silver nitrate into the tissues was exceedingly slow and incomplete.

The technique of Leschke was repeated, but it yielded no satisfactory results. Control tissues from animals which had not received iodide gave histologic deposits of silver indistinguishable from those obtained from animals which had been injected with iodide. The surface of the blocks revealed far more precipitate than the central portion; this is evidence that iodide diffused out of the tissue much more rapidly than the reagent entered. We must, therefore, consider the conclusions of Leschke invalid.

Palladous chloride, although forming a dense brown and highly insoluble palladous iodide, also precipitates tissue proteins very rapidly and penetrates poorly. Perfusion with palladous chloride yielded precipitates of brown palladous iodide only near the blood vessels. The results obtained were similar to those previously reported(10).

Mercuric acetate, mercurous nitrate, and lead nitrate were also employed with unsatisfactory results. When the mercurous salt was employed, the granular deposit of reduced mercury masked any yellow iodide precipitate that may have formed. Perfusion with a saturated solution of thallous chloride yielded no demonstrable precipitate. The procedure of Hintzelmann(3) gave unsatisfactory results. Perfusion with thallous acetate resulted in the precipitation of yellow granules of thallous iodide, but these were limited to the areas immediately adjacent to the blood vessels, which indicated poor penetration and post-mortem diffusion of the iodide from the tissue.

In all these attempts to demonstrate iodides histochemically the slow penetration of the precipitating reagents led to unsatisfactory results.

3. *Attempts to precipitate relatively colorless insoluble iodides with conversion to colored precipitates after sectioning*

Conversion of lead, mercurous, mercuric, and thallous iodide into the dark brown palladous iodide, after sectioning the tissue, confirmed the results reported just above.

Diphenyl-iodonium acid sulphate ($[(C_6H_5)_2I]^+HSO_4^-$) reacts like an inorganic ion and resembles thallium salts in precipitating iodides. Iodonium iodide is about one-fifth as soluble as thallous iodide in water, but is quite soluble in alcohol, acetone, formalin, and acid solutions. Hence it is necessary to employ frozen sections. The application of diphenyl-iodonium acid sulphate was suggested by Prof. Julius Stieglitz and was prepared under his direction. A 3 per cent solution of the iodonium salt was perfused at various intervals after injection of potassium iodide. Frozen sections, cut with a dry knife, were dropped directly into a concentrated solution of palladous chloride. The resulting dark brown precipitate was more or less diffuse, but far more localized and concentrated than after any other technique. Perfusion through the pancreatic duct of a rabbit injected with potassium iodide gave results resembling those obtained by the direct perfusion of palladous chloride. This reagent penetrated the tissues in a more satisfactory manner, but the results still were not ideal.

Copper sulphate, with any one of a number of reducing agents, or cuprous chloride, acidified with a few drops of hydrochloric acid, form a white precipitate of cuprous iodide (solubility about 1 part in 150,000 at 18°C.). This precipitate can be easily converted into the dark brown palladous iodide. Although copper sulphate perfused well, the cupric ion is readily reduced to metallic copper in the tissues. In the presence of a reducing agent, reduction occurs on standing at room temperature or more rapidly with heat. When the solution becomes very concentrated (as occurs during dehydration of the block of tissue) reduction of the copper is marked and especially is this so when such reducing agents as

potassium bisulphite, sodium sulphite, sulphurous acid, or sodium thiosulphate are added. Since any metallic copper yields a black precipitate of metallic palladium with palladous chloride, the method is not specific for iodide.

Cuprous chloride is too insoluble to yield a solution sufficiently concentrated for perfusion. Cuprous chloride can be dissolved in hydrochloric acid or in concentrated potassium or sodium chloride solutions; dilution of the perfusion reagent by blood and tissue fluids, however, causes an immediate precipitation of cuprous chloride in the blood vessels.

CONCLUSIONS

1. After a thorough, critical application of a large number of chemical methods for precipitating iodides to histochemical technique, we are convinced that no technique thus far presented is wholly satisfactory.

2. Repetition of the techniques previously suggested by Stieglitz, Leschke, and Hintzelmann failed to confirm their observations.

3. The inadequacy of all the reactions studied can be attributed to the extremely rapid diffusibility of the iodide ion or the failure of the precipitating reagent to penetrate the tissue proteins rapidly enough. For histochemical purposes the reaction must not only yield an extremely insoluble precipitate, but the introduction of the precipitating reagent must be almost instantaneous. As heavy metals are required for the precipitation of iodide ions, the tendency of these metals to precipitate protein and thus block their penetration is an apparently insurmountable difficulty.

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THE MACERATION AND RESORPTION OF FETUSES IN THE RAT

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SIX TEXT FIGURES AND ONE PLATE (FOUR FIGURES)

The voluminous literature on this subject records principally isolated findings in material obtained fortuitously from animals and from the operating room. The phenomenon of fetal retrogression has long been noted and commented upon (Müller, 1847), but little experimental evidence on this subject has been published. Schlesinger ('04) reviewed much of the earlier literature on autolysis in his study of fetal and newborn rabbits; he concluded that the autolytic activity of the fetal tissues reaches its maximum at birth, declining with increasing age. Mendel and Leavenworth ('08) considered that the enzymes necessary for autolytic tissue disintegration were present early in the embryo, but that they were held in check by the normal environment and reactions of the cells. Koebner ('10) reported that even the bony skeleton of rabbit fetuses may be completely resorbed. More recently, Meyer ('19) described the resorption of early human conceptuses and concluded that the fetus was "usually the first member of the conceptus to disappear." The autolysis was usually complete. He attributed this to the low resistance of the fetus to the enzymes originating within its own tissues. Meyer also comments on the very considerable period over which small fetuses may retain their outline, although extensively macerated. Kuntz ('20, '22) and Castle ('28) have described necrotic fetuses in rabbit uteri.

In the present paper an experimental approach to the problem of the maceration of fetuses in the albino rat is made, together with a study of the effect of fetal death on the mother. The maternal leucocytic reaction to necrosing (resorbing) fetuses in utero was particularly studied.

METHODS

Fetal death was brought about by two methods: 1) by manual separation of the placenta from the endometrium, and, 2) by removal of the fetuses from the uterus and placing them free within the maternal peritoneal cavity. In the first method the cervix was loosely looped with a ligature in such a manner as to prevent abortion without in any way interfering with the maternal circulation. In the second method the uterus was incised and the contained fetuses, with their placentae, removed and placed in the abdominal cavity of the mother without attempt at subsequent repair of the uterus. Autopsy of these animals (see end of section on maternal leucocytes) revealed that the uterus healed autonomously and reassumed its normal size and position.

Daily and differential leucocyte counts were made of the maternal blood before and for 10 to 16 days following operation. Blood samples were drawn in all cases from the caudal vein of the tail. At the end of the experimental period, the animals were sacrificed and the uteri with their contents, or, in the case of (2), the fetuses within the peritoneal cavity, were removed and fixed in 5 per cent formalin solution. The fixed material was subsequently sectioned, stained with hematoxylin and eosin, and used for microscopic study.

EFFECT OF FETAL AGE ON MORTALITY

Of the twenty experimental animals used, nine lived throughout the period of experimentation and eleven died in from 1 to 3 days. In seeking a possible cause for this high mortality, the crown-rump length of the fetuses (as recorded at operation) was apparently significant. It was found that death of the mother almost invariably took place in the case

of operations which involved the older (larger) fetuses. The results are summarized in the following table.

Number of cases	Average C-R length of fetuses, mm.	Extremes, mm.	Result
9	21	16-30	Mother survived
11	30	22-35	Mother died in 1 to 3 days

One would be led to relate maternal death, therefore, to the death in utero of the older (greater C-R length) fetuses. The formation of toxic substances in greater quantity or concentration by the necrosing tissues of the larger fetuses may be postulated. The occurrence of convulsions in one animal calls to mind the fact that eclamptic convulsions in the human usually occur late in pregnancy when the fetus is large (Williams, '30). Whatever the cause of maternal death in these experimental cases, it is invariably associated with the presence of large dead fetuses either within the uterus or the peritoneal cavity.

Autopsy of animals which succumbed failed to disclose the cause of death. In all cases the abdomen was clear of infection, and the amount of peritoneal exudate was small. In these animals the total leucocyte count, nevertheless, rose until the time of death. The neutrophiles, in contrast, showed a considerable initial increase followed by a fall to the pre-operative level.

EFFECT OF FETAL DEATH ON THE MATERNAL LEUCOCYTES

1. *Leucocyte count in control animals*

Three pregnant control animals were used. In one animal daily total and differential leucocyte counts were made for a period of 14 days in order to determine the normal variation in the leucocytic content of the blood. The results may be summarized as follows:

Average total count (cells per cu.mm.)	Extremes	Average neutrophiles	Extremes
9673	7600-12800	23 per cent	14-35 per cent

The daily variation in these values appears to be quite large. Differences up to 100 per cent in the neutrophile count were

apparently of common occurrence; yet, on autopsy, the animal was found to be in a healthy condition with no sign of infection. It has been noted by Kindred and Corey ('30) that the pregnant rat does not differ from the non-pregnant rat as regards the leucocytic content of the blood.

Two more pregnant animals were selected and laparotomy and repair were performed. Similar determinations were made on these animals. Their average leucocyte count was 10,200 cells. Figure 1 shows the daily fluctuations in the leucocyte count of one of these animals. Autopsy revealed no evidence of infection nor could any sign of parasitization be found.

It is concluded from the above that a considerable variation may occur in the leucocytic content of the blood of the pregnant rat. Following laparotomy and repair, there is a definite reaction of the white cells which soon subsides and the count, both total and differential, returns to normal.

2. Effect on animals in which fetal death was produced by placental separation

Total leucocytes. The average leucocyte count for five animals treated as above was 8760 per cubic millimeter. From this number there was a rise to an average of 13,533, and later a fall to 7666 cells at the time the animals were sacrificed (10 to 16 days after operation). The count rose sharply after operation, and then fell to approximately the normal level in an average time of about 6 days (fig. 2). The picture presented by the total leucocyte count in these animals is essentially the same as that seen in control animals after performing laparotomy and repair only (compare figs. 1 and 2).

Differential count. The differential leucocyte count does not offer any criteria to distinguish it from differential counts on control animals following simple laparotomy and repair. Figures 3 and 4 show graphically a comparison between differential counts on control and experimental animals over a 10-day period. In these cases the neutrophiles

of the control animal show more pronounced reaction than in the animal containing dead fetuses in its uterus. In neither case are the monocytes significantly affected. It is concluded that the retention and maceration of dead fetuses in utero does not involve maternal leucocytic variation to a degree demonstrable by the methods employed.

3. Effect on animals in which fetal death was produced by removal of fetuses to the peritoneal cavity of the mother

Total leucocytes. The total leucocyte count in these (4) animals increased markedly from the time of operation until the rats were sacrificed for withdrawal and preservation of the contained fetuses. The initial leucocyte count averaged 7366 cells per cubic millimeter. With rather wide fluctuation, there was a rise from this number to an average of 13,300 cells at the end of the experimental period (fig. 5).

Differential count. Daily differential counts revealed the fact that the neutrophiles rose sharply immediately following operation and maintained their high level until the animals were sacrificed. The lymphocytic values rose and fell in inverse proportion to the neutrophiles as revealed by a percentage count. The monocytes remained relatively unaffected. Figure 6 illustrates the changes graphically.

It was concluded that necrosing fetal tissue lying in the peritoneal cavity of the mother brings about a marked leucocytic reaction. The maceration of such fetuses may be attributed, in part at least, to the attack of the maternal leucocytes. The apparent tolerance of the maternal system for dead fetal tissue is hence only exhibited while that tissue remains within the uterus; and the maternal leucocytic reaction to the fetal tissue within the peritoneal cavity is similar to that usually elicited by any foreign body. The uterus may act in a protective capacity against the toxic substances liberated by the necrosing (resorbing) fetuses, while the dead fetal tissue within the cavity reacts in an irritating and toxic manner. The process of maceration and resorption in utero of dead fetuses in the rat does not appear, therefore, to involve extensive leucocytic intervention.

NECROPSY FINDINGS

The following typical necropsy findings have been selected to illustrate the condition of animals when sacrificed after 10 to 16 days of fetal retention.

1. Animals in which the fetuses were killed by placental separation

No. E-3. Uterus normal in appearance; the loose ligature was found intact; there was no evidence of infection, with the exception of a small amount of clear peritoneal exudate.

No. E-5. The peritoneum was clear and without evidence of infection. There were no adhesions; the uterus was without signs of necrosis, and normal in appearance. The viscera were not discolored. The uterine vessels were enlarged, but normal in appearance as compared to the usual appearance of the blood vessels in pregnant animals.

No. E-11. Uterus normal in size and position when compared with notes taken at operation, but there was slight discoloration. There was considerable peritoneal exudate. The other viscera appeared normal.

2. Animals in which the fetuses were removed to the maternal peritoneal cavity

No. E-6. Laparotomy wound area clear; no peritoneal exudate; no adhesions. The fetal remains were small and macerated. The uterus had returned to normal size; the uterine section was visible as a series of white dots.

No. E-7. The remains of all fetuses were found; they were in a macerated condition. There was no peritoneal exudate. Some adhesions had formed around the uterus; the uterus had healed completely.

COMPARISON OF LEUCOCYTE COUNTS IN CONTROL AND
EXPERIMENTAL ANIMALS

Fig. 1 Daily leucocyte count in pregnant control (rat no. C-1), illustrating the effect of laparotomy and repair only.

Fig. 2 Daily leucocyte count in pregnant animal following fetal death by placental separation (rat no. E-5).

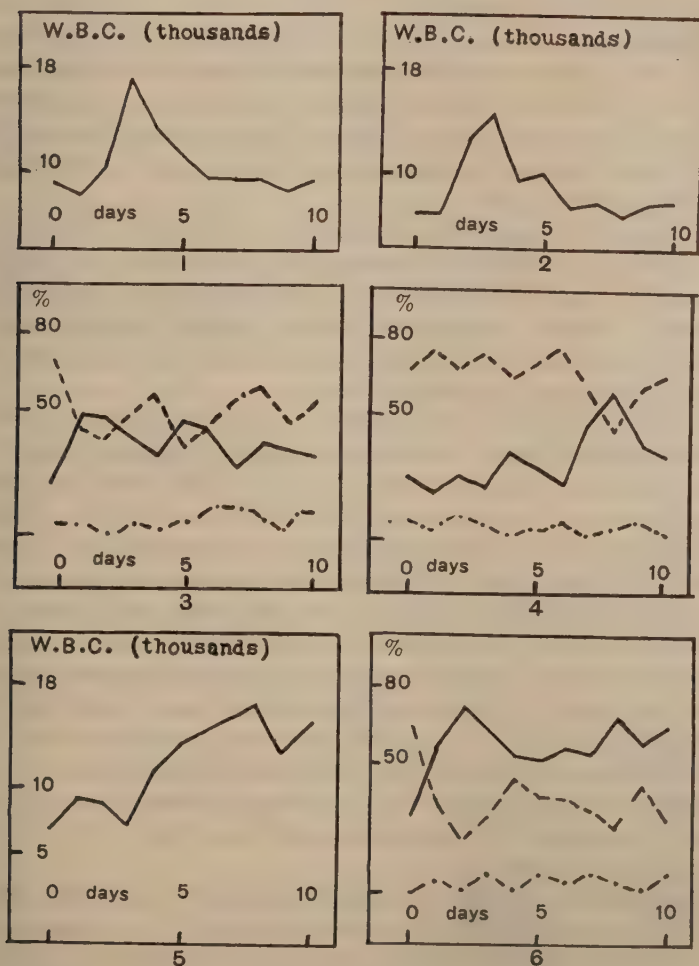
Fig. 3 Differential count in pregnant control (rat no. C-1); laparotomy and repair only. —, neutrophiles; ---, lymphocytes; - - - - -, monocytes.

Fig. 4 Differential count following fetal death by placental separation (rat no. E-5).

Fig. 5 Daily leucocyte count in pregnant animal following transference of fetuses from uterus to peritoneal cavity of the mother (rat no. E-6).

Fig. 6 Differential count in the same animal.

No. E-9. The fetal remains had adhered to the omentum and intestinal peritoneum; on the omentum they were embedded in fat. The wound was clear of infection; the uterus was normal in size and had assumed its original position.



Figures 1 to 6

EFFECT OF INTRAUTERINE DEATH OF FETUSES AND OF ECTOPIC PREGNANCY IN HUMAN MATERIAL

In an attempt to correlate findings in the rat with conditions obtaining in man, reference was made to the records of the University Hospital.

Intrauterine death of fetus. Of these cases, nineteen were selected which were uncomplicated by other pathological conditions as revealed by the records. The average leucocyte count in these cases was 10,170 cells per cubic millimeter with 6200 and 19,000 as the extremes. The average is seen to be within normal limits.

Ectopic pregnancy (unruptured). In thirteen cases without other complication, the average leucocyte count was 10,050 with 6000 and 21,000 cells per cubic millimeter as the extremes. Again the average number of cells present is well within the normal range, and the similarity of these findings to the foregoing is clearly evident.

Ectopic pregnancy (ruptured). In seventeen cases uncomplicated by infection or other pathological findings, the average leucocyte count was found to be 18,256 cells per cubic millimeter. The extremes were 8400 and 51,000. These figures make it evident that, in man, the presence of a fetus within the peritoneal cavity results in a marked increase in leucocytes in the blood stream, as was noted in the rat. In the above cases the absence of sufficient differential counts made it impossible to ascertain the type of cell accounting for this increase. It appeared from the meager material that was available that the increase was in the neutrophiles. In all the above cases the leucocyte count on admission to the hospital was tabulated for the above averages, since treatment, operative or otherwise, might obviously introduce extraneous factors.

In both the rat and man, therefore, there is apparently little if any reaction of the maternal leucocytes to the presence of a dead fetus in the uterus. On the other hand, the presence of dead fetal tissue in the peritoneal cavity results in a definite blood cell reaction, expressing itself in the production

of large numbers of leucocytes, especially of the neutrophile type. The uterus may thus be said to act in a protective manner against the necrosing fetal tissue present in its lumen.

EXAMINATION OF THE UTERUS, FETAL MEMBRANES, AND FETUS

1. Cases in which fetal death was produced by placental separation

Uterus. Uteri which contained necrosed fetal tissue appeared normal in respect to both muscle layers and mucosa in the majority of the specimens studied. Endometrial disintegration appeared in a few cases where resorption was almost complete. Figure 7 is considered particularly illuminating, since the fetuses in this case have been almost completely resorbed. In this specimen the uterine wall was not necrotic, but exhibited areas of neutrophile and monocyte infiltration at irregular intervals. The mucosa was absent and the lumen of the uterus contained only fetal debris together with masses of phagocytic cells. The vascularity of the uterus is considerable, but not demonstrably greater than that seen in the normal pregnant uterus.

Fetal membranes. The fetal envelope is in most cases intact. This observation is in agreement with those of Meyer ('19). In some cases, as exemplified by figure 8, the membranes exhibit perforation. This cannot be considered as an artefact, since beneath such perforations the fetal tissues have undergone considerable disintegration. The fetal membranes may persist intact even in badly necrosed fetuses. This fact, together with the observation immediately above, points to the fetal membranes as the major protective factor against uterine disintegration under the influence of enzymes liberated by the fetus. One is inclined to look on the membrane barrier as a wholly mechanical one. The placenta, unlike the amnion, undergoes necrosis early in the macerative process.

Fetus. The striking persistence of the fetal outline even in extensively disintegrated fetuses corroborates the observations of Meyer. Fetuses exhibiting almost perfect outline

show marked visceral disintegration. The first disintegrative changes apparently take place in the viscera—the liver (fig. 9) and gastro-intestinal tract suffering first. This fact points to the fetal origin of the macerative process. The process of resorption in the rat fetus is extremely variable in rate. During the same length of time (10 days) in utero, some fetuses remained in astonishingly good condition, while others showed extensive tissue breakdown. The process of fetal retrogression requires several days for its initiation; even after 10 days some of the tissue cells (e.g., in skeletal muscle) appear to be in excellent condition. The cartilage cells are apparently the last to die, as would be expected. As is usual in such cases, considerable amounts of calcium have been deposited in the cartilage. That this is not normal bone seems certain from the fact that it appears in fetuses at an age which does not normally show any ossification whatever (Corey, '32). For example, fetuses 16 mm. long at operation showed extensive calcium deposition after 10 days' retention, while the first traces of ossification that the author has been able to demonstrate in normal fetuses appeared at the 22 to 23 mm. stage.

The process of maceration and resorption of dead fetuses begins by the slow death of the fetal tissue at a rather inconstant rate. Autolytic degeneration begins probably first in the liver and digestive tract. These necrotic changes then spread to the spleen, pancreas, etc., and ultimately involve the muscular (skeletal) tissue (fig. 10). The superficial covering (skin) disintegrates at about the same time as the muscles. The maternal neutrophiles appear to be involved only in the removal of the fetal remains (fig. 7). These cells are very numerous within the lumen of the uterus after fetal resorption is almost complete, but the blood neutrophiles fail to increase to any demonstrable extent. These 'scavenger' neutrophiles are probably migrants from the vascular system, but their connective tissue origin cannot be ruled out. Their function appears to be solely the removal of fetal debris.

2. Cases in which fetal death was caused by displacing the fetuses from the uterus to the maternal peritoneal cavity

As in the preceding type of experiment, necrosis of fetal tissues varied in rate, but did not appear to take place more rapidly in those cases in which the amnion was left intact. Fetuses placed free in the maternal cavity, with amnion removed, were more completely resorbed than their litter mates with the membranes left intact. The fetal membranes appeared thus to serve as a barrier against both invasion of the mother by lytic enzymes of fetal origin, and phagocytes or lytic substances generated by the mother. Maceration of these fetuses is initiated by enzymes of fetal origin—internal disintegration taking place first and in approximately the same manner as in previous cases (section 1). The dermal elements in fetuses without the protection of the amnion invariably showed great disintegration. The rise in the maternal neutrophils may be looked upon in these cases as due to the usual 'foreign body' reaction to toxic substances within the peritoneal cavity.

CONCLUSIONS

Near-term fetuses, which have been either separated from the maternal blood supply in the uterus or placed free in the maternal peritoneal cavity, bring about the early death of the mother.

Intrauterine death of the fetus produced no demonstrable effect on the leucocytic content of the maternal blood throughout the experimental period employed (10 to 16 days).

Extrauterine death of the fetus evoked a profound leucocytic increase on the part of the mother.

The experimental conditions observed in the rat appear to be comparable to pathological conditions found in man, particularly as regards the leucocytic reaction of the mother.

Maceration and resorption of fetuses in the rat is initiated by enzymes of fetal origin, involving the liver and gastrointestinal tract. Subsequent disintegration involves the skeletal muscle and cartilaginous tissues.

The uterus and fetal membranes act as barriers against toxic substances liberated by the necrosing fetal tissues.

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PLATE

PLATE 1

EXPLANATION OF FIGURES

7 Resorption of 16 mm. fetus almost complete (16 days after placental separation). The uterine musculature (Ut.) is not necrotic. Many phagocytes (N.) are seen in the uterine cavity. Disintegration of the fetus (F.) is complete. $\times 180$.

8 The amnion (Am.) has perforated. The outer layers of the fetal skin (Epi.) have undergone marked degenerative changes (10 days after placental separation). $\times 360$.

9 Advanced degeneration of the liver of a 26 mm. fetus 10 days after placental separation. $\times 360$.

10 Skeletal muscle from a 20 mm. fetus after 12 days in the maternal peritoneal cavity. $\times 360$.

